SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF PRETREATED SWITCHGRASS USING THERMOTOLERANT IMB STRAINS OF *KLUYVEROMYCES MARXIANUS*

By

BRIAN A. FAGA

Bachelor of Science in Mechanical Engineering

Purdue University

West Lafayette, Indiana

2004

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2009

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF PRETREATED SWITCHGRASS USING THERMOTOLERANT IMB STRAINS OF *KLUYVEROMYCES MARXIANUS*

Thesis Approved:

Dr. Mark Wilkins

Thesis Adviser

Dr. Danielle Bellmer

Dr. Nurhan Dunford

Dr. Niels Maness

Dr. A. Gordon Emslie

Dean of the Graduate College

ACKNOWLEDGMENTS

First and foremost, I want to thank my parents, Richard and Gayle, and the rest of my family and friends for their continued support and encouragement throughout my studies and research. My accomplishments and achievements would not mean nearly as much as they do if I could not share them with you.

I would like to extend my deepest gratitude to my advisor, Dr. Mark Wilkins, for providing me the opportunity, the funding, and the guidance that made my research possible. I would also like to thank my committee members, Dr. Danielle Bellmer, Dr. Nurhan Dunford, and Dr. Niels Maness as well as other faculty members of the Biosystems and Agricultural Engineering Department at Oklahoma State University that have provided me with the insight and direction needed to complete my thesis. I would like to acknowledge the Oklahoma Bioenergy Center and the Oklahoma Agricultural Experiment Station Team Initiative Program for funding and supporting this work.

Thanks are also in order for Dr. Ibrahim Banat for allowing the use of his microorganisms in my experiments and to Robert Ingraham and Jarrod Major at the Advanced Technology Research Center for their assistance and support.

TABLE OF CONTENTS

Chapter **Page**

Chapter **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

LIST OF TABLES

Table **Page**

CHAPTER I

INTRODUCTION

The United States imports more than two thirds of the oil it consumes, costing the economy hundreds of billions of dollars every year (Energy Information Administration 2007). The price of a barrel of oil has become increasingly volatile, reaching an all time high of $147 in July of 2008 and dropping to below $40 less than six months later (Anonymous 2009). The refining and burning of oil is also associated with the release of greenhouse gases and other pollutants that may cause climate change and environmental damage. Developing renewable transportation fuels, such as ethanol, that can be produced in the United States will be a factor in alleviating the political, economic, and environmental issues associated with petroleum.

The U.S. government has enacted a Renewable Fuels Standard that mandates an increase in the amount of ethanol blended into gasoline from 9 billion gallons in 2008 to 36 billion gallons in 2022 (Environmental Protection Agency 2008). Most automobiles in the U.S. can be powered by gasoline containing 10% ethanol, a blend known as E10, and many newer vehicles can run on blends up to 85% ethanol (California Energy Commission 2004). In 2007, 6.5 billion gallons of ethanol were produced in the United States (Renewable Fuels Association 2009), and essentially all ethanol produced in the U.S. is made from corn (Urbanchuk 2006). However, it is estimated that corn ethanol production will be maximized at 15 billion gallons per year (US Department of

Agriculture Economic Research Service 2005). The production of ethanol from corn has raised issues over diverting food crops to produce transportation fuel and the amount of fossil fuels, fertilizer, and water required to grow corn. Brazil has successfully replaced 40% of its petroleum needs with ethanol produced from sugar cane, but sugar cane can be grown in only a few areas of the United States (Knight 2006). Other feedstocks and processes need to be developed to efficiently and economically produce the remaining 20 billion gallons of ethanol per year that will be required by the Renewable Fuels Standard.

Ethanol can be produced from lignocellulosic biomass including trees, sawdust, municipal solid waste, agricultural residue such as corn stover, and dedicated energy crops such as switchgrass or miscanthus (Huang et al. 2009; Wiselogel et al. 1996). Lignocellulosic biomass is much more resistant to preprocessing and hydrolysis required to produce fermentable sugars for ethanol production than corn starch or sugar cane (Mosier et al. 2005). However, ethanol from cellulosic biomass offers numerous advantages over petroleum derived fuels and corn ethanol. A study by Argonne National Laboratory done in 1999 determined that replacing gasoline with a blend of 15% gasoline and 85% cellulosic ethanol known as E85 would reduce petroleum use by 70% (Wang et al. 1999). Cellulosic ethanol facilities powered mainly by residual lignin instead of fossil fuels reduce net greenhouse gas emissions by almost 100% and reduce fossil energy use by 75% (Wang et al. 1999). Using E85 made with corn ethanol results in the same 70% reduction in petroleum use but only reduces net greenhouse gas emissions by 25% and fossil energy use by 42% (Wang et al. 1999). Compared to corn, cellulosic biomass crops can be grown using lower quality soils and locations, less water, and less fertilizer (Kim and Dale 2005).

Ethanol can be produced from cellulosic biomass in a four step process that includes pretreatment, hydrolysis, fermentation, and dehydration (Mosier et al. 2005). Hydrolysis and fermentation can be performed concurrently in a process known as simultaneous saccharification and fermentation (SSF) (Takagi et al. 1977). SSF utilizes enzymes instead of chemicals such as acids to depolymerize structural carbohydrates, mainly cellulose and hemi-cellulose, into fermentable sugars. SSF reduces equipment costs by performing the hydrolysis and fermentation in a single reactor and eliminates the need for expensive materials capable of withstanding strong acids or other chemicals (Wright 1988). A diagram of the overall cellulosic ethanol process is shown in Figure 1.1. A major challenge in improving the SSF process is matching the temperature conditions required for optimum performance of the enzyme and the fermenting microorganism (Bollok et al. 2000). The optimum temperature for cellulase enzymes is higher than can be tolerated by common yeasts used for industrial ethanol production (Ballesteros et al. 2004; Kiran Sree et al. 2000).

A number of thermotolerant yeast strains have been identified that have potential for use in the SSF process at elevated temperatures. *Kluyveromyces marxianus* yeast strains have been used in a number of studies with promising results (Ballesteros et al. 2004; Hughes et al. 1984; Lark et al. 1997; Nonklang et al. 2008). In particular, five strains of *K. marxianus* identified by Banat (1992) have shown favorable fermentation results at temperatures between 40 and 50 ^{o}C. The purpose of this work was to evaluate the effectiveness of these thermotolerant strains in the SSF of pretreated switchgrass at 45 ^{o}C. The best performing strain was selected and then used in investigations to determine optimum SSF conditions for increasing ethanol yields.

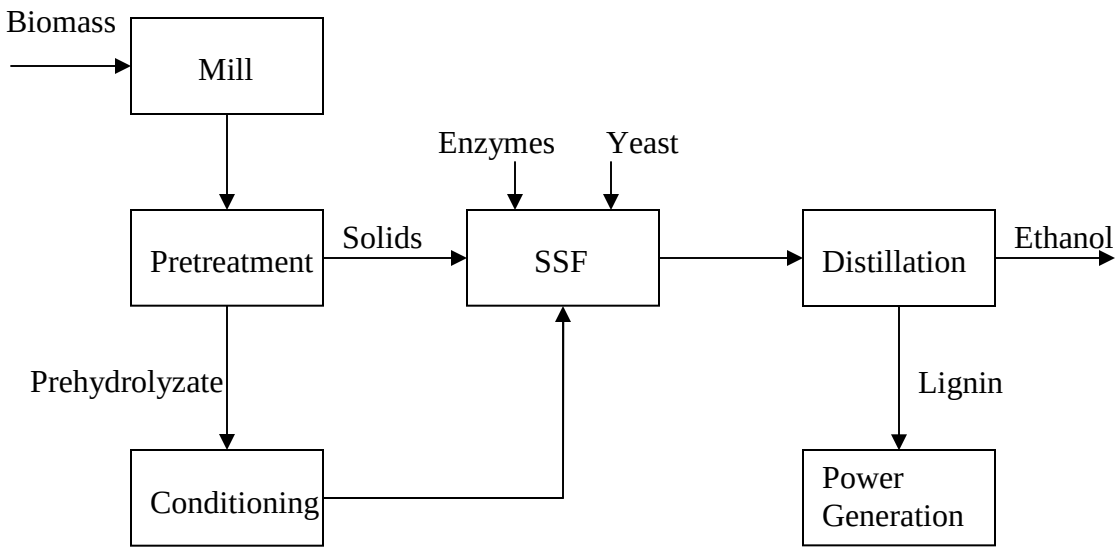

Figure 1.1 Enzymatic Cellulosic Ethanol Process

CHAPTER II

OBJECTIVES

The objectives of this research were as follows:

1. Screen five strains of *Kluyveromyces marxianus* yeast for ethanol production in an SSF process at 45 ºC with pretreated Kanlow switchgrass and Fibrilase enzyme and compare the results with *Saccharomyces cerevisiae* D_5A in the same SSF process performed at 37 ºC.
2. Explore the effects of reduced cellulase enzyme loading on fermentation and ethanol production by *K. marxianus* IMB 3 and *S. cerevisiae* D_5A in an SSF process with pretreated Kanlow switchgrass.
3. Explore the effects of pH on ethanol production and fermentation time by *K. marxianus* IMB 3 and *S. cerevisiae* D_5A in an SSF process with pretreated Kanlow switchgrass.

CHAPTER III

REVIEW OF LITERATURE

3.1 Switchgrass

Switchgrass (*Panicum virgatum*) is a C4 perennial sod-forming grass that is native to the plains of North America (Weaver 1968). It is capable of growth in many environments such as prairies, marshes, and wooded areas and is naturally resistant to many pests and plant diseases. It has high water use efficiency and grows well in unirrigated plots (Bransby 2004; Koshi et al. 1982). It is cultivated as an agricultural crop for forage and erosion control (Hitchcock et al. 1951). Switchgrass is attractive for forage because it produces large amounts of biomass compared to relatively low inputs of water and nutrients. It is also capable of growth on poor soils that would not support traditional row crops (Moser and Vogel 1995). Switchgrass is compatible with current farming practices because it can be planted and harvested with existing agricultural equipment (McLaughlin et al. 2002).

Switchgrass occurs in Lowland and Upland varieties. Upland switchgrass is generally shorter and found in dryer climates that do not experience water runoff. Lowland switchgrass is taller, up to 12 feet in height, and is suited for wetter conditions (Bransby 2004; Gunter et al. 1996). Lowland varieties of switchgrass such as Kanlow or Alamo can produce 16 Mg ha^{-1} yr^{-1} in established unirrigated stands (McLaughlin and

Walsh 1998). A study done in Iowa with 20 varieties of switchgrass concluded that Kanlow produced the most biomass of the varieties tested (Lemus et al. 2002). Switchgrass also has positive effects on soil conditions because it does not require annual replanting and produces a large root system that can penetrate the soil down to ten feet (Anderson and Coleman 1985; Bransby 2004). Through its large root system, which can be equivalent in mass to the portion of the plant above ground, switchgrass increases soil carbon and organic matter content (Anderson and Coleman 1985; Lynd et al. 1991). In fact, switchgrass can potentially sequester 1.7 Mg of carbon ha^{-1} (McLaughlin et al. 2002). These qualities make switchgrass attractive as a potential bioenergy feedstock.

The United States Department of Energy's Biofuels Development Program (BFDP) chose switchgrass as a model bioenergy crop because it produces high yields of biomass, requires relatively low inputs (Sanderson et al. 1996), and is able to grow in a variety of geographic locations and soil conditions. The natural range of switchgrass extends from Canada to Mexico (McLaughlin and Walsh 1998). Switchgrass can serve as a bioenergy crop by being used as fuel in a traditional combustion boiler or as a feedstock for production of ethanol and other chemicals (Lemus et al. 2002). Switchgrass plots can be established in three years, with yields in the first two years being only 33-66% of expected full yield. In the first two years, resources are devoted to growing the root system. The root system provides many of the benefits of switchgrass in the seasons after establishment. After the initial establishment years, switchgrass can be harvested once or twice a year depending on water and fertilizer input (McLaughlin and Kszos 2005). Efforts to increase biomass yields through nitrogen application and irrigation have shown success, but the upper limits of yields have most likely not yet been

met. Application of nitrogen to existing switchgrass stands in Iowa from 1998 to 2002 resulted in a 37% increase in biomass yield (Lemus et al. 2008). A study on Alamo switchgrass in Texas yielded 8 Mg ha^{-1} in an unirrigated plot compared to 20 Mg ha^{-1} in an irrigated plot (Mitchell et al. 2008). Switchgrass is an ideal crop for bioconversion to ethanol due to its ability to produce large amounts of biomass with low inputs and its positive soil and environmental benefits.

3.1.1 Switchgrass Composition

The cell walls of switchgrass are composed of structural polysaccharides and organic compounds. Cellulose, hemicellulose, and lignin account for more than 70% of the harvested biomass on a dry basis (db) (Chen et al. 2002; Dien et al. 2006). The composition of switchgrass can vary as follows: 31 to 42% cellulose, 25 to 31% hemicellulose, 10 to 17% lignin, 5 to 11% ash, and 10 to 14% extractives, which include soluble carbohydrates and crude protein (Dien et al. 2006; Sarath et al. 2007; Wiselogel et al. 1996). As the switchgrass plant matures, the percentage of lignin and total carbohydrates increases (Dien et al. 2006). The mineral content of ash has been found to contain Al, Ca, K, P, Si, Mg, Cl and S (El-Nashaar et al. 2009). Lignin has the highest energy content of the compounds found in switchgrass, however, cellulose and hemicellulose contain the carbohydrates that can be fermented into ethanol (Wiselogel et al. 1996). For switchgrass to be used as an energy crop for either thermochemical processes or bioconversion to ethanol, the amounts of cellulose, hemicellulose, and lignin should be considered.

The structural carbohydrates in switchgrass include glucose, xylose, galactose, arabinose, and mannose residues (Suryawati et al. 2008). Cellulose, the main structural

material of the cell wall of switchgrass, is an unbranched crystalline polymer of β(1→4) linked D-glucose molecules typically 100 to 20,000 glucose units in length that begins to become soluble when the chain is less than 12 glucose units (Gardner and Blackwell 1974; Klemm et al. 1998; Zhang and Lynd 2004). Hemicellulose in switchgrass is a branched linear polymer with a xylan backbone and side chains of L-arabinose, D-galactose, D-mannose and glucoronic acid. Xylan is a polymer of β(1→4) linked D-xylose molecules (Wong and Saddler 1992).

Lignin is the second most common organic compound after cellulose and contains as much as 30% of the organic carbon available on earth (Boerjan et al. 2003). It is a polymer of cross-linked phenolic compounds derived from the phenylpropanoid pathway that does not have a defined chemical structure. This network acts as a binder of the structural carbohydrates and provides the cell wall with strength and rigidity. It is also an important component of the plants vascular system aiding the transport of water and nutrients throughout the plant (Adler 1977; Boerjan et al. 2003).

3.2 Pretreatment

The complex network structure of cellulose, hemicellulose, and lignin in lignocellulosic materials such as switchgrass is a major barrier to the efficient production of chemicals and fuels on an industrial scale (Mosier et al. 2005). Separation of each of these fractions into individual feedstock streams is crucial for the economical and efficient utilization of biomass (Mok and Antal 1992). A diagram of the goals of pretreatment is shown in Figure 3.1 in which lignin is disrupted, hemicellulose is solubilized, and cellulose is exposed. In the case of cellulosic ethanol, the enzymatic

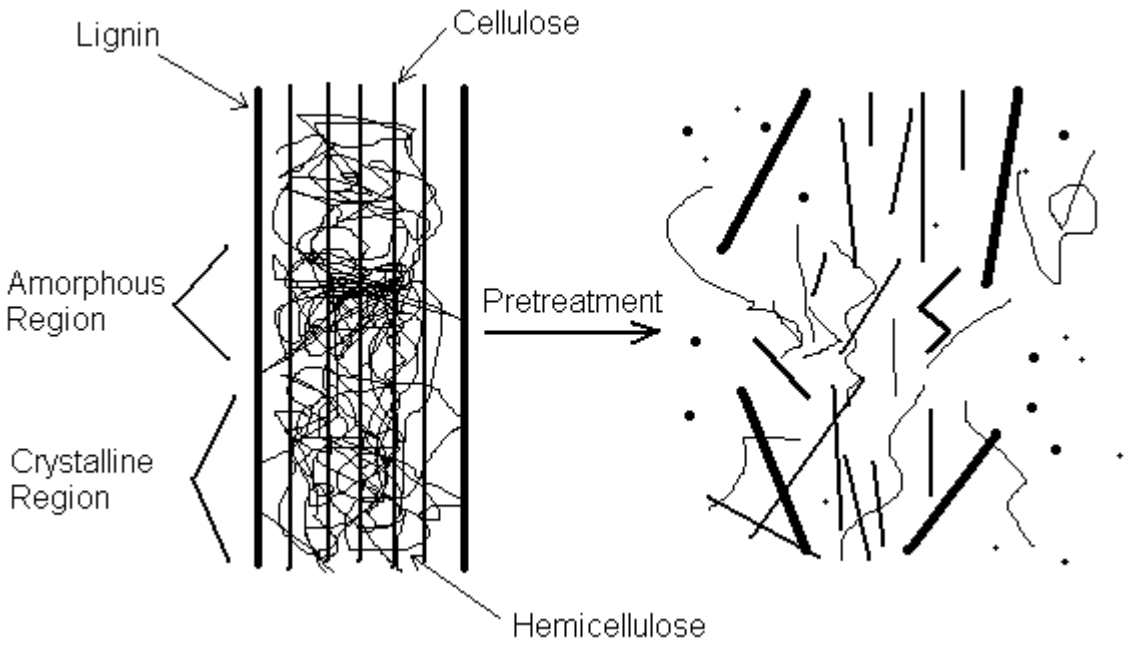

Figure 3.1 Effect of Pretreatment on Lignocellulosic biomass - Adapted from Hsu et al. (1980).

digestibility of cellulose must be increased to ensure maximum utilization of carbohydrates by fermenting organisms and decrease the amount of expensive enzymes needed for hydrolysis (Allen et al. 1996; Mosier et al. 2005; Weil et al. 1998). Enzymatic hydrolysis of cellulose in raw biomass typically yields less than 20% of the potential glucose (Wright 1988). Therefore, a pretreatment step is necessary to remove hemicellulose, open the lignin structure, and decrease the crystallinity of cellulose (Chang et al. 2001; Fan et al. 1982). The National Research Council (1999) also indicated that effective pretreatment methods should minimize the energy consumed by the process, maximize the recovery of pentose sugars, and minimize the formation of compounds that will inhibit fermentation. Many methods of pretreatment have been studied with varying amounts of success. Regardless of the method employed, the pretreatment step is likely to be among the most expensive and crucial steps in the process (Lynd et al. 1996).

3.3 Pretreatment Technologies

Pretreatment techniques can be classified as physical, chemical and combinations of each (Hsu 1996; McMillan 1994). Physical methods include mechanical comminution (milling, grinding, size reduction), steam explosion, and hydrothermolysis. In many cases, physical methods are used as an initial step prior to chemical treatment (Hsu 1996; Millett et al. 1979; Mosier et al. 2005). Many different chemical treatments have been investigated for pretreatment including acids, bases, and solvents. Cellulose solvents including H_2O_2, ozone, $FeCl_3$, Al_2SO_4, glycerol, dioxane, phenol and ethylene glycol have shown large improvements in the effectiveness of enzymatic hydrolysis of cellulose to glucose (Wood et al. 1988). 90% of cellulose in corn stalks was converted to glucose

when treated with cellulose solvents (Ladisch et al. 1978). Strong acids such as H_2SO_4 and HCl have also shown to be effective at dissolving hemicellulose, disrupting lignin, and decrystallizing cellulose. Although these chemical methods have shown success, they are generally too expensive to scale up (Mosier et al. 2005).

3.3.1 Lime

Lime (CaOH) is mixed with water to form an alkaline slurry. The slurry is applied to the biomass for a period of hours to days. The process can be done at ambient temperatures, but temperatures above 85 ºC reduce the pretreatment time (Mosier et al. 2005). Lime pretreatment works by removing lignin and deacetlylating hemicellulose (Chang and Holtzapple 2000). Switchgrass pretreated by 0.1 g lime / g dry switchgrass in 9 mL water / g dry switchgrass at 100 to 120 ºC for two h solubilized 26% of the xylan and 29% of the lignin in the switchgrass. 90% of the glucan remained in the residue (Chang et al. 1997). Corn stover, another widely available cellulosic substrate, was pretreated with lime at 0.075 g lime / g dry biomass and 5 g water / g dry biomass at 120 ºC for four h. Subsequent enzymatic hydrolysis converted 88.0, 87.7, and 92.1% of the glucan, xylan, and arabinan to monomeric sugars. Lime also has the benefit of being relatively inexpensive and recoverable as calcium carbonate, which can be regenerated in a kiln (Chang et al. 1997; Chang et al. 1998).

3.3.2 Dilute Acid

Dilute sulfuric acid at concentrations of 0.5 to 2.0% can be mixed with lignocellulosic biomass and heated above 160 ºC for no more than a few minutes as an effective pretreatment method (Lee et al. 1999). Acid depolymerizes hemicellulose chains into oligomers and monomers and eventually degradation products such as

furfural (Lee et al. 1999). The resulting residue has reduced hemicellulose and has increased porosity and surface area for enzymatic hydrolysis (Brownell and Saddler 1984). The process is stopped before cellulose hydrolysis begins by neutralizing the acid (Lee et al. 1999). In one previous study, switchgrass was pretreated using 1.2% sulfuric acid at 180 ºC. The resulting residue was used in an SSF process and 90.3% of the cellulose was converted to glucose, cellobiose, and ethanol (Chung et al. 2005). A major drawback to the dilute acid process is the corrosive nature of sulfuric acid, which requires expensive reactor materials and neutralization chemicals (Mosier et al. 2005).

3.3.3 AFEX

Ammonia fiber explosion (AFEX) exposes lignocellulosic biomass to aqueous ammonia under pressure and then rapidly relieves the pressure causing damage to the physical structure of the biomass (Mosier et al. 2005). Ammonia depolymerizes lignin and removes hemicellulose (Dale et al. 1996). Cellulose exposed to ammonia swells and the crystallinity is reduced (Lin et al. 1981). Ammonia fiber explosion reduces the formation of degradation products by utilizing relatively mild conditions, T<90 ºC and pH<12 (Mosier et al. 2005).

3.3.4 Steam Explosion

Steam explosion is a physical pretreatment method in which biomass is exposed to steam at temperatures above 160 ºC typically without the presence of any other chemicals. After a specified period of time, the pressure is quickly released (Heitz et al. 1991; Laser et al. 2002). The rapid decompression ends and cools the reaction and disturbs the physical structure of the biomass (Mosier et al. 2005). During the initial heating, acetyl groups from hemicellulose form acetic acid further hydrolyzing the

hemicellulose (Brownell and Saddler 1984). The rapid hydrolysis of hemicellulose leads to low recovery of xylan and the formation of fermentation inhibitors (Allen et al. 2001; Laser et al. 2002). Steam explosion is not effective at solubilizing lignin (Bobleter 1994). Due to the high energy content of steam, higher solids loading can be achieved, however, for the same reason, carbohydrate degradation is typically higher with steam explosion (Allen et al. 2001). Steam exploded corn fiber loaded at 70% solids and heated to 215 ºC lead to the solubilization of 37% of the biomass. When used in an SSF process with 15 FPU cellulase / g cellulose, 90% of the cellulose was converted to ethanol (Allen et al. 2001).

3.3.5 Hydrothermolysis

Hydrothermolysis, or pressurized liquid hot water, is another physical pretreatment method that utilizes only the chemical properties of heated water to break down the structure of lignocellulosic biomass (Mosier et al. 2005). Water is heated from 180 to 230 ºC under pressure to remain in the liquid state. The heated water is exposed to biomass in a batch or flow through style reactor for 5 to 20 min (Allen et al. 2001; Liu and Wyman 2005; Mok and Antal 1992; Suryawati et al. 2008; Weil et al. 1998). At 200 ºC, water becomes acidic with a pH of 5.0 (Weil et al. 1997). In this acidic environment, acetyl groups are cleaved from hemicellulose to form acetic and other organic acids. These organic acids further hydrolyze hemicellulose into oligmomers and monomers (Mosier et al. 2005). Hydrothermolysis has also been shown to dissolve all of the hemicellulose and between one-third to two-thirds of lignin in biomass while retaining most of the cellulose (Antal 1996). Liquid hot water cannot completely delignify biomass because components of lignin can recondense after the heating sequence of

pretreatment (Bobleter and Concin 1979). Hydrothermolysis is an effective pretreatment method because it completely removes hemicellulose, reduces lignin, and maintains cellulose. It also does not require chemicals for pretreatment and the expensive reactor materials associated with some of these chemicals.

Studies of hydrothermolysis pretreatment of biomass have been done using different reactor configurations, including batch and flow-through (Mok and Antal 1992; Weil et al. 1998). Using a tubular percolating reactor, Mok and Antal (1992) pretreated ten species of lignocellulosic biomass by hydrothermolysis at temperatures between 200 and 230 ºC. The hot water was pumped through the biomass by an HPLC pump at 34.5 MPa for 0 to 15 min at a flow rate of 1 mL / min. During the process, 40 to 60% of biomass was solubilized. This method dissolved all of the hemicellulose from each species of biomass and recovery of monomers resulting from hemicellulose was on average greater than 90%. Greater than 80% of cellulose was retained in the residual solids and 35 to 60% of lignin was solubilized (Mok and Antal 1992). In another study, corn fiber at 4.4% solids was pretreated by pH-controlled hydrothermolysis in a batch 2 L Parr reactor (Weil et al. 1998). The reactor was heated to 200, 220, 240, and 260 ºC. Heat up time took between 50 and 60 min. KOH was added to the reactor to maintain the pH above 5.0. The addition of KOH is used to prevent auto-catalyzed acid reactions that will degrade cellulose. Following the pretreatment, tap water was circulated in an internal cooling coil, dropping the temperature to 180 ºC within 2 min. Over 70% of the biomass was solubilized during pretreatment regardless of the temperature, and hemicellulose was fully dissolved under all conditions. At 240 ºC, cellulose content was increased to 47.3% compared to 17.5% for untreated corn fiber. Enzymatic hydrolysis of

corn fiber pretreated at 220 ºC with KOH added resulted in 84% conversion of cellulose to glucose (Weil et al. 1998). Hydrothermolysis can be an effective pretreatment for ethanol production from biomass because it removes hemicellulose and lignin and recovers a high percentage of the monomer pentoses while retaining a high percentage of cellulose.

Several researchers have performed simultaneous saccharification and fermentation on biomass that has been pretreated by hydrothermolysis. Sugar cane bagasse, aspen chips, and mixed hardwood flour was pretreated by hydrothermolysis in an immersed percolation reactor at 220 ºC and 5 MPa for 2 min (van Walsum et al. 1996). Complete hemicellulose removal occurred with over 80% recovery of pentosans and less than 10% solubilization of cellulose. SSF of the residual solids resulted on average in greater than 90% conversion of the cellulose to ethanol within 75 h (van Walsum et al. 1996). Laser (2002) pretreated sugar cane bagasse in a 25 L batch reactor with water at 170 to 230 ºC for up to 46 min. The best performing pretreatment condition was at 220 ºC for 2 min with a 5% solids concentration. Under these conditions xylan recovery and conversion of cellulose to ethanol by SSF were both greater than 80% (Laser et al. 2002).

3.4 Enzymatic Hydrolysis of Cellulose

One method of hydrolyzing cellulose into glucose for microbial fermentation is using extracellular cellulase enzymes produced by certain filamentous fungi. The cellulase enzyme systems of the *Trichoderma* species are the most commonly used and have been utilized in a number of studies (Philippidis et al. 1993; Zhang and Lynd 2004). The cellulolytic enzyme systems are mixtures of three types of enzymes with different

specific functions (Figure 3.2). Two of these enzymes, 1,4-β-D-glucan exoglucanase and 1,4-β-D-glucan endoglucanase, breakdown large molecules of insoluble cellulose into small soluble oligomers, mainly cellobiose (Philippidis et al. 1993). To begin reducing the polymerization of cellulose, endoglucanase hydrolyzes random glucosidic bonds in cellulose and opens the crystalline structure into long chains. Exocellulase adsorbs onto these linear chains of cellulose and moves along them releasing cellobiose units into solution (Klemm et al. 1998; Zhang and Lynd 2004; Zhou et al. 2009). The third enzyme, β-D-glucosidase, splits cellobiose into individual glucose monomers (Philippidis et al. 1993; Zhou et al. 2009).

Many factors determine the effectiveness and efficiency of the cellulase enzyme mixture. The optimum temperature for cellulose hydrolysis by enzymes from the *Trichoderma* species is between 30 and 50 ºC (Ballesteros et al. 2004; Zhang and Lynd 2004). Structural characteristics of cellulose such as degree of polymerization, crystallinity, available surface area and lignification can decrease the effectiveness of the enzyme system. Effective pretreatment methods are designed to increase the susceptibility of biomass to enzymatic hydrolysis by changing these characteristics (Ballesteros et al. 2004; Philippidis et al. 1993; Zhang and Lynd 2004). The presence of cellobiose and glucose can slow the rate of cellulose and cellobiose hydrolysis through product inhibition of cellulase and β-glucosidase enzymes, respectively (Philippidis et al. 1993). Varying the ratio of endocellulase, exocellulase, and β-glucosidase from that found in natural systems can increase the effectiveness of the cellulase systems. An optimized cellulase mixture of endocellulases, exocellulases, and β-glucosidase enzymes from *Trichoderma viride* released glucose 2.1 times faster than the natural mixture of the

same enzymes (Zhou et al. 2009). Increasing the effectiveness of cellulase enzyme systems by pretreatment and optimization is crucial because the cost and cellulolytic efficiency of enzymes are major economic factors that are slowing the commercialization of the cellulosic ethanol process (Galbe and Zacchi 2002; Himmel et al. 1999; Nieves et al. 1998).

3.5 Simultaneous Saccharification and Fermentation

Ethanol production from cellulose is a two step process requiring hydrolysis followed by fermentation (Saddler et al. 1982). Performing enzymatic hydrolysis and fermentation in separate, distinct stages is known as separate hydrolysis and fermentation (SHF) (Wright 1988). During the hydrolysis stage, product inhibition caused by accumulation of sugars released from cellulose results in long reaction times (Philippidis et al. 1993). The high concentration of sugars makes the hydrolyzate susceptible to contamination by unwanted organisms (Wright 1988). An alternative to SHF, simultaneous saccharification and fermentation (SSF), can be used to produce ethanol directly from cellulose using cellulase enzymes and yeast in a single stage reactor (Takagi et al. 1977). Utilizing SSF, fermentable sugars released by hydrolysis are quickly converted to ethanol by the yeast, which reduces product inhibition of the cellulase enzymes (Philippidis et al. 1993) and reduces the probability of contamination (Lastick et al. 1983). Performing the reaction in a single vessel also reduces capital costs and operating expenses (Wright 1988).

SSF can be performed with traditional ethanol producing yeast such as *Saccharomyces cerevisiae* (Deshpande et al. 1983; Duff and Murray 1996), but the temperature must be kept between 25 and 30 ºC for the yeast to remain active (Kiran Sree

et al. 2000). At these temperatures, the effectiveness of the cellulase enzymes is diminished because their optimum temperature ranges from 40 to 50 ºC (Ballesteros et al. 2004; Zhang and Lynd 2004). In order to maximize the rate of cellulose hydrolysis, a thermotolerant yeast capable of fermentation above 40 ºC should be used in the SSF process (Szczodrak and Targonski 1988). Performing the SSF at higher temperatures also results in energy savings by reducing the cooling requirements needed to remove the heat created by metabolic activities (Banat et al. 1998).

3.5.1 SSF at Increased Temperatures with Thermotolerant Yeast

Many different yeast strains have been screened for their ability to produce ethanol at elevated temperatures (Ballesteros et al. 1991; Spindler et al. 1989; Szczodrak and Targonski 1988). Spindler et al. (1989) performed SSF of Sigma-cell 50 cellulose substrate with *Candida lusitaniae, Candida brassicae, Candida acidothermophilum,* and *Saccharomyces uvarum* at 37, 41, and 43 ºC. *T. reesei* cellulase enzyme was loaded at 13 IU/g substrate for all SSFs (Spindler et al. 1989). For all of the yeast, viability decreased as temperature increased, and *S. uvarum* did not grow at 43 ºC. The conversion rate of cellulose to ethanol ranged from 55 to 71 % and decreased as temperature increased for all of the yeast, therefore, these strains are not good candidates for high temperature SSF of cellulose (Spindler et al. 1989). Szczodrak and Targonski (1988) evaluated a total of 58 yeast strains for their ability to produce ethanol from glucose, cellobiose, galactose, mannose, xylose, and arabinose at 40, 43, and 46 ºC. The yeasts were from the genera *Aureobasidium, Candida, Cryptococcus, Fabospora, Kloeckera, Kluyveromyces, Pachysolen, Pichia, Saccharomyces, Schizosaccharomyces, Trichosporon,* and *Torulopsis* (Szczodrak and Targonski 1988). The best performing yeast, *F. fragilis* CCY

51-1-1, produced 56, 56, and 35 g ethanol/L at 40, 43, and 46 ºC, respectively, from 140 g glucose/L. To determine inhibitory effects, this yeast was then used in a glucose fermentation with 400 FPU/L of *T. reesei* cellulase enzyme. The presence of cellulase had only a minimal effect on glucose utilization and ethanol production (Szczodrak and Targonski 1988). Ballesteros et al. (1991) performed a similar study with 27 strains of yeast from the genera *Candida, Saccharomyces, and Kluyveromyces.* After 48 h, *K. marxianus* and *K. fragilis* performed the best in glucose fermentations at 45 ºC producing 21.9 and 20.8 g ethanol/L respectively from media containing 50 g glucose/L. In SSFs of Solka-floc at 42 ºC, both of these strains produced 50 % of theoretical ethanol after 78 h (Ballesteros et al. 1991).

Various strains of *S. cerevisiae* have been researched for compatibility with high temperature SSF processes. Krishna et al. (1999) pretreated *A. leptopus* (Linn) leaves with alkaline hydrogen peroxide in preparation for SSF with *S. cerevisiae* NRRL-Y-132 and *T. reesei* cellulase supplemented with β-glucosidase. The optimum conditions were found to be 40 ºC, 10 % solids (w/v), 100 FPU/g substrate of cellulase, and pH 5.1. After 72 h, the ethanol concentration under these conditions was 2.6% (w/v) (Krishna et al. 1999). Lime pretreatment was used to prepare switchgrass and corn stover for SSF with *S. cerevisiae* D_5A (Chang et al. 2001). The SSFs were performed at 38 ºC, pH 5.0, and 25 FPU/g cellulose Spezyme-CP cellulase enzyme. The SSFs resulted in 72 % of cellulose from switchgrass and 62 % of cellulose from corn stover being converted to ethanol (Chang et al. 2001). Kiran Sree et al. (2000) isolated four thermotolerant strains of *S. cerevisiae* named VS1, VS2, VS3, and VS4 from soil samples taken from a thermal power plant in India. All four strains were found to be able to produce ethanol from

glucose at 44 ºC. The best performing strain, VS3, produced 75, 60, and 58 g ethanol/L from 150 g glucose/L at 30, 40, and 44 ºC (Kiran Sree et al. 2000). Edgardo et al. (2008) screened 11 strains of *S. cerevisiae* for the purpose of finding a suitable yeast for SSF at temperatures between 35 and 45 ºC. While all 11 strains were able to ferment glucose media at 35 and 40 ºC, only two strains, IR2 and IR2*, grew at 42 ºC. No strains grew at 45 ºC (Edgardo et al. 2008). The strain IR2 produced 77 % of theoretical ethanol yield at 40 ºC and was selected to be used in SSF at 40 ºC with bleached kraft pulp and organosolv pretreated *P. radiata* chips. The SSF contained 10% (w/v) substrate and 20 FPU/g substrate Celluclast 1.5L cellulase enzyme supplemented with 20 IU/g substrate β-glucosidase. SSF of bleached kraft pulp and organosolv pretreated *P. radiata* chips with IR2 produced 62 and 73 % of theoretical ethanol after 72 h, respectively (Edgardo et al. 2008).

Strains of *K. marxianus* have been identified that ferment glucose at temperatures from 35 to 52 ºC making them good candidates for high temperature SSF processes (Banat et al. 1992; Banat et al. 1998; Hughes et al. 1984). Lark et al. (1997) performed SSF on recycled paper sludge with *K. marxianus* ATCC 36907 at 38 ºC. The yeast was capable of glucose fermentation at temperatures up to 43 ºC, however 38 ºC was chosen for SSF to maintain cell viability for the 72 h fermentation period (Lark et al. 1997). SSFs were prepared with 10 % (w/v) paper sludge, 8 FPU/g dry paper sludge cellulase enzyme, and a buffer with pH 5.0. At these conditions *K. marxianus* ATCC 36907 produced 32 to 35 g ethanol/L by converting approximately 72 % of the cellulose in the paper sludge to ethanol (Lark et al. 1997). Ballesteros et al. (2004) used steam explosion to pretreat poplar, eucalyptus, wheat straw, sweet sorghum bagasse, and *B. carinata*

residue in preparation for SSF with *K. marxianus* CECT 10875 at 42 ºC. The SSF was prepared with substrate at 10 % (w/v) dry solids and 15 FPU/g substrate Celluclast 1.5L cellulase enzyme. Ethanol concentrations after 72 to 82 h reached 16 to 19 g ethanol/L. Cellulose to ethanol conversion was 71.2, 62.5, 62.5, 60.9, and 68.1 % for poplar, eucalyptus, wheat straw, sweet sorghum bagasse, and *B. carinata* residue, respectively (Ballesteros et al. 2004). Nonklang et al. (2008) identified a strain of *K. marxianus*, DMKU3-1042, that is capable of growth at 49 ºC and ethanol production from glucose at 45 ºC. This study also showed that this strain can utilize substrates for growth that *S. cerevisiae* cannot, including cellobiose, xylose, xylitol, arabinose, glycerol, and lactose. This strain has not yet been utilized in SSF experiments but is expected to provide advantages similar to other thermotolerant *Kluyveromyces* yeasts (Nonklang et al. 2008).

3.5.2 Thermotolerant IMB strains of *Kluyveromyces marxianus*

Banat et al. (1992) isolated and identified five strains of *K. marxianus* from samples taken at an Indian distillery. The five strains, named IMB1, IMB2, IMB3, IMB4, and IMB5, were all capable of growth on glucose media at 52 ºC. At 50 ºC, fermentation of 14 % (w/v) glucose resulted in 5.1 to 5.5 % (w/v) ethanol. The highest ethanol production by IMB1 and IMB3 was 6.7 and 6.5 % (w/v) at 40 ºC. At 45 ºC, IMB2, IMB4, and IMB5 produced 7.2, 6.8, and 7.0 % (w/v) ethanol respectively (Banat et al. 1992). Further study by Banat and Marchant (1995) showed that all five strains are capable of growth on lactose, whey permeate, cellobiose, and xylose at 45 ºC. The ability to utilize cellobiose and xylose is important for yeasts that are to be used in SSF of lignocellulosic feedstocks. These yeast strains also grew relatively fast (μ = 0.18 to 0.19 h^{-1}) on glucose substrate under anaerobic conditions at 40 ºC compared to *S. cerevisiae* (μ

= 0.3 to 0.4 h^{-1}). The strains were able to produce ethanol concentrations up to 95 g/L and ethanol production was not affected until ethanol concentration reached 75 g/L (Banat and Marchant 1995). A number of other studies have reported promising results using IMB strains for high temperature fermentation of multiple substrates (Barron et al. 1994; Brady et al. 1994; Fleming et al. 1993; McCabe et al. 1995; Simpson et al. 1995). Singh et al. (1998) attempted full scale fermentations of molasses with IMB3 at a distillery in India that normally uses *S. cerevisiae*. These fermentations were conducted without the use of the distillery's typical cooling system allowing the fermentation to reach temperatures up to 42 ºC. The results showed that IMB3 resulted in ethanol concentrations of 6.0 to 7.2 % (w/v), which was equivalent to the typical yields achieved by the distillery's strain of *S. cerevisiae*. It was also found that the use of IMB3 resulted in shorter fermentation times than *S. cerevisiae*, 16 to 20 h compared to 22 to 26 h (Singh et al. 1998).

These promising results have lead to IMB3 being studied for use in the SSF of cellulosic materials (Barron et al. 1997; Boyle et al. 1997; Nilsson et al. 1995; Suryawati et al. 2008; Suryawati et al. 2009). The SSF of pulverized barley straw at 45 ºC with IMB3 and 2 % (v/v) *T. reesei* cellulase enzyme produced low ethanol concentrations (Boyle et al. 1997). Improved results were seen after pretreating the barley straw with 5 M NaOH. Ethanol concentrations from SSFs containing 2, 4, and 6 % (w/v) pretreated barley straw were 3.9, 8.0, and 12.0 g/L respectively after 70 h. Based on estimates of barley straw cellulose content, the percent of theoretical ethanol yield in these experiments ranged from 95 to 98 % (Boyle et al. 1997). Barron et al. (1997) performed a similar experiment at 45 ºC with IMB3 by supplementing distillery spent wash with

NaOH pretreated straw. The percentage of cellulose converted to ethanol from SSFs containing 2, 4, and 6 % (w/v) solids was 75, 76, and 86 % respectively (Barron et al. 1997).

Suryawati et al. (2008) and Suryawati et al. (2009) used IMB4 in experiments to optimize hydrothermolysis pretreatment and the subsequent SSF conditions of Kanlow switchgrass. Switchgrass was milled through a 13 mm screen and pretreated by hydrothermolysis in a 1 L Parr reactor to investigate the most effective conditions (Suryawati et al. 2009). A 10 % (w/w) solids mixture of dry switchgrass and water with a total mass of 600 g was sealed in the stirred reactor vessel. The vessel was heated to 190, 200, or 210 ºC, and the reactor temperature was maintained for 10, 15, or 20 min. The reactor was immediately cooled in an ice bath. Glucan content in the residual solids tended to increase as hold time increased. The highest glucan content, 64.3%, was achieved at 190 ºC and hold time of 20 min. The glucan content of the native switchgrass in this work was 36.6%. Xylan recovery decreased with hold time and temperature. The maximum xylan recovery, 73.1%, was achieved at 190 ºC and hold time of 10 min. Formation of fermentation inhibitors hydroxymethylfurfural (HMF) and furfural in the prehydrolyzate was less than 1 g/L under all pretreatment conditions. The residual solids from each pretreatment condition were used in an SSF process at 45 ºC with *K. marxianus* IMB 4 and 15 FPU/g glucan of Fibrilase cellulase enzyme to evaluate the effect on ethanol production. The glucan content in each SSF was 41 g/L (Suryawati et al. 2009). The optimum temperature and time combination for pretreatment was determined by the residue that resulted in the highest percentage conversion of glucan to ethanol. Switchgrass pretreated at 200 ºC for 10 min had a glucan content of 51.3% (db)

and resulted in the highest conversion of glucan to ethanol, 74.2% (Suryawati et al. 2009). To determine the optimum temperature for SSF of pretreated switchgrass by IMB4, Suryawati et al. (2008) performed SSF at 37, 41, and 45 ºC. A 50 mM citrate buffer was used to provide an initial pH of 4.8. The effect of pH was also studied by performing SSF at 45 ºC with initial pH of 5.5. All results were compared to SSF with *S. cerevisiae* D_5A at 37 ºC and initial pH of 4.8 (Suryawati et al. 2008). The highest conversion of glucan to ethanol by IMB 4, 78 %, occurred at 45 ºC with initial pH of 5.5 after 96 h. Equivalent ethanol production was not achieved by the *S. cerevisiae* D_5A SSF with pH 4.8 buffer until 168 h. The highest ethanol yield achieved by IMB4 SSF with initial pH of 4.8 was 69 % at 45 ºC after 72 h. In all SSFs performed at 41 and 45 ºC, fermentation ceased between 72 and 96 h. However, the cellulase continued hydrolyzing glucan, resulting in residual glucose concentrations reaching as high as 5.2 g/L at the end of the SSF (Suryawati et al. 2008).

3.6 Effects of pH in Simultaneous Saccharification and Fermentation

Organic acids produced during pretreatment or fermentation can inhibit growth and fermentation characteristics of yeast (Narendranath et al. 2001; Palmqvist and Hahn-Hägerdal 2000). At low pH, acetic and lactic acid have fungicidal effects on yeast (Neal et al. 1965). As the pH falls below the pKa of acetic acid (pKa = 4.74), the increasing concentration of the undissociated form of the acid has inhibitory effects (Freese et al. 1973). In its undissociated form, acetic acid can permeate the cell membrane of the yeast. Once inside the cell, the acid dissociates and interferes with the metabolic activities (Kashket 1987). Narendranath et al. (2001) showed that the specific growth rate of *S. cerevisiae* decreased exponentially as the concentration of acetic acid was

increased. These experiments were performed at 30 ºC and pH 4.5. Decreases in glucose consumption and ethanol production were seen when acetic acid concentration was as low as 0.1 % (w/v) and the minimum inhibitory concentration (MIC) was found to be 0.6 % (w/v) (Narendranath et al. 2001).

Bajpai and Margaritis (1987) fermented Jerusalem artichoke extract with *K. marxianus* UCD(FST)55-82 at 35 ºC and initial pH values of 3, 4, 5, 6, and 7 in order to optimize biomass production and ethanol production. The fermentation at initial pH 5.0 resulted in the highest growth rate, 0.35 h^{-1}, and the highest final ethanol concentration, 44.82 g/L. Fermentations with an initial pH higher or lower than 5.0, produced noticeably lower growth and ethanol production rates. Viegas et al. (1989) found similar results by studying the inhibitory effects of octanoic and decanoic acids produced by *K. marxianus* during ethanolic fermentation of Jerusalem artichoke juice. The toxic effects of these byproducts increased as the pH was lowered from 5.4 to 3.0, indicating that the undissociated form of these acids is more harmful to the metabolic activities of the yeast. Production of β-galactosidase enzyme by *K. marxianus* CDB 002 has also been shown to be influenced by pH. Furlan et al. (2001) monitored production of β-galactosidase in a sugar-cane molasses medium and found that the highest enzyme production occurred with initial pH of 5.5. In SSF experiments with *K. marxianus* IMB 4, Suryawati et al. (2008) found that an initial pH of 5.5 using a sodium citrate buffer resulted in a theoretical ethanol yield of 79% compared to 70% with a pH 4.8 buffer. At initial pH of 5.5, fermentation continued at least 24 h longer than at pH 4.8 and resulted in 40% less acetic acid production (Suryawati et al. 2008). Maintaining the pH of SSFs above the

pKa of acetic acid and other organic acids may prevent inhibition by undissociated acid resulting in improved yeast performance and higher ethanol yield.

CHAPTER IV

METHODS AND MATERIALS

4.1 Switchgrass Preparation and Native Compositional Analysis

Samples of Kanlow switchgrass (*Panicum virgatum* var. Kanlow) grown at the Oklahoma State University Plant Sciences Research Farm were milled through a 2 mm screen in a Thomas-Wiley mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA.) Soluble extractives were removed from switchgrass samples prior to determination of structural carbohydrates and lignin content. The two step National Renewable Energy Laboratory (NREL) extraction procedure (Sluiter et al. 2005) was done automatically using an Accelerated Solvent Extractor (Model 300, Dionex Corporation, Sunnyvale, CA) utilizing water and ethanol as solvents. Each solvent was used in three extraction cycles performed at 1500 psi and 100 ºC. Water-extracted samples were evaporated in an oven at 40 ºC for 48 h. Ethanol-extracted samples were evaporated in a RapidVap N2 Evaporation System (Labconco Corporation, Kansas City, KS) at 500 mbar and 40 ºC for 24 h. The mass of the extractives were recorded after drying.

Following extraction, the residual switchgrass was analyzed for structural carbohydrates, lignin, acetyl-groups, and ash content using NREL procedures LAP 002 and 005 (Sluiter et al. 2004a; Sluiter et al. 2004b). An Isotemp programmable muffle furnace (Fisher Scientific, Dubuque, IA) was used in LAP 002 and 005. The samples for analysis of carbohydrates and acetyl groups were filtered through a 0.2 µm syringe tip

filter into a 1.5 mL HPLC sample vial and capped. Analyses of carbohydrates and organic acids were done by HPLC with refractive index detection (RID) (Agilent 1100 Series, Santa Clara, CA). 20 µL of each sample were analyzed. Carbohydrate determination samples were analyzed on an Aminex HPX-87P carbohydrate column at 85 ºC with a mobile phase of deionized water pumped at 0.6 mL/min for 35 min (Sluiter et al. 2004b). The samples were analyzed for cellobiose, glucose, xylose, galactose, arabinose, and mannose. Acetyl group content samples were analyzed for acetic acid on an HPX-87H organic acid column at 60 ºC with a mobile phase of 0.01 N H_2SO_4 pumped at 0.6 mL/min for 50 min (Sluiter et al. 2004b). Acid-soluble lignin (ASL) content in switchgrass was determined using a UV-VIS spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA) set at a wavelength of 205 nm. The wavelength, 205 nm, and absorbtivity, 110 L/g-cm, for determining ASL in switchgrass were taken from Thammasouk (1997).

4.2 Switchgrass Pretreatment by Hydrothermolysis

Switchgrass was pretreated by hydrothermolysis in a 1-L Parr pressure reactor (Figure 4.1) (Parr Series 4520, Parr Instrument Company, Moline, IL). The reactor was filled with 60 g of dry switchgrass and 540 g of deionized water. The completely sealed reactor was then heated to 200 ºC while being agitated at 150 rpm. The heating time took between 32 and 36 min. The temperature was then held at 200 ºC for 10 min (Suryawati et al. 2009). The reactor was then immediately cooled in an ice bath until the temperature fell below 30 ºC. The solid and liquid fractions were then separated by vacuum filtration through Whatman #5 filter paper. The solids were rinsed and vacuum filtered with 2 L of deionized water to remove any residual soluble sugars or compounds. A sample,

Figure 4.1 1-L Parr Pressure Reactor used for Hydrothermolysis of Switchgrass at 200 °C.

approximately 6 g, of the washed residual solids was dried in an oven at 105 ºC to determine the mass of dry solids recovered after pretreatment (Sluiter et al. 2004c). The structural carbohydrate and lignin composition of the pretreated switchgrass was then determined by NREL LAP 002 (Sluiter et al. 2004b) as described previously. The carbohydrate content determined in this procedure was reported as glucan and xylan instead of cellulose and hemi-cellulose because the structural polysaccharides are hydrolyzed to monomer sugars to be measured. Therefore, glucan and xylan represent the total glucose and xylose content, respectively, in the material. This pretreatment process was repeated multiple times, and the pretreated solids were combined in order to accumulate enough material to perform the required experiments.

4.3 Determination of Cellulase Enzyme Activity

The cellulase used for these experiments was a commercially available enzyme named Fibrilase (Iogen, Ottawa, Canada). In order to add the proper amount of enzyme, the activity of the cellulase must be determined prior to use in SSF. The procedure to determine cellulase activity used was the standard filter paper assay (Ghose 1987). Strips of Whatman #1 filter paper with mass of approximately 50 mg were hydrolyzed by buffered solutions containing enzyme at different concentrations (1:150, 1:175, and 1:200) with the goal of releasing glucose at slightly more than and slightly less than 2 mg/mL. Following incubation for 60 min at 50 ºC, dinitrosalicylic acid (DNS) reagent was added to stop hydrolysis and combine with reducing sugars to provide a colorometric indicator of glucose concentration. The absorbance of each enzyme concentration was measured at 540 nm on a UV-Vis spectrophotometer. A calibration curve of glucose concentration versus absorbance was created with stock solutions of glucose at different

concentrations. The amount of glucose released by each enzyme concentration was then used to determine the activity of the cellulase in filter paper units per mL of enzyme (FPU/mL).

4.4 Yeast Inoculum Preparation

Inoculum cultures of *K. marxianus* IMB 1, IMB 2, IMB 3, IMB 4, IMB 5 and *S. cerevisiae* D_5A were grown in YPD media containing 10 g/L yeast extract, 20 g/L peptone, and 50 g/L dextrose (Dowe and McMillan 2001). A loopful of each yeast strain was taken from a slant and used to inoculate 100 mL of YPD media in a 250 mL baffled flask. The media had been sterilized by filtration through a 0.22 µm filter (500 mL Sterile Bottletop Filter, Corning Life Sciences, Big Flats, NY) and the flask was sterilized by autoclave at 121 ºC. The inoculum flask was topped with an aerobic stopper (Bugstopper, Whatman Inc., Florham Park, NJ) and incubated on a rotary shaker (MaxQ 4450, Thermo Scientific, Dubuque, IA) at 250 rpm for 16 h. *K. marxianus* strains were incubated at 45 ºC and *S. cerevisiae* D_5A was incubated at 37 ºC. Samples of the inoculum were taken and diluted with deionized water to achieve an absorbance between 0.5 and 1.0 at a wavelength of 605 nm on a UV-VIS spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA). The absorbance and dilution of each inoculum were then recorded and used to determine optical cell density in the inoculum. Optical density of 0.5 is equivalent to 0.14 g/L of *K. marxianus* cells and 0.20 g/L of *S. cerevisiae* D_5A cells (Suryawati et al. 2009). Based on the cell density of the inoculum and the desired optical density of cells of 0.5 in the experiment flasks, the proper volume of inoculum was taken from the inoculum flask and placed in a 50 mL centrifuge tube. The yeast cells were separated from the inoculum media by centrifuging at 3500 rpm for 5 min. The

supernatant was decanted and replaced with deionized water. The cells were resuspended, centrifuged, and decanted a second time. The cells were resuspended in deionized water to make an optical density of 50. To provide an initial optical density of 0.5, 1 mL of concentrated cells was added to each shake flask SSF, and 15 mL was added to the bioreactor SSF.

4.5 Simultaneous Saccharification and Fermentation

In order to evaluate the performance of all five IMB strains and *S. cerevisiae* D_5A, three identical SSFs were performed with each strain following NREL LAP 002 (Dowe and McMillan 2001). SSFs with IMB strains were performed at 45 ºC, and SSFs with *S. cerevisiae* were performed at 37 ºC. All SSFs were prepared by loading 250 mL baffled flasks with pretreated switchgrass containing 4.2 g of glucan. The flask and switchgrass were then autoclaved at 121 ºC. Following sterilization of the flask, 10 mL of filter sterilized (500 mL Sterile Bottletop Filter, Corning Life Sciences, Big Flats, NY) nutrient media containing 20 g/L KH_2PO_4, 20 g/L $(NH_4)_2SO_4$, 10 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L yeast extract, and 1 g/L $MnSO_4$; 5 mL of 1 M sodium citrate buffer with pH 5.5; 15 FPU/g glucan Fibrilase enzyme; and 1 mL of concentrated yeast cells in deionized water to provide an initial OD of 0.5 (Banat et al. 1992; Dowe and McMillan 2001). Deionized water that had been autoclaved at 121 ºC was added to make the total mass 100 g. The flasks were capped with stoppers fitted with one way air valves to allow the flasks to vent without allowing air in and incubated for 168 h while being rotated at 130 rpm. 1.5 mL samples were taken at 0, 4, 24, 48, 72, 96, 120, 144, and 168 h. The samples were frozen for later analysis. After 168 h, the pH of each SSF was measured and recorded (Dowe and McMillan 2001). In order to account for ethanol or other products resulting from

fermentation of the nutrient media or the enzyme mixture in each SSF, one fermentation was performed with each yeast strain and identical components and conditions as the experimental SSFs excluding pretreated switchgrass.

To explore the effects of decreased cellulase enzyme loading, SSFs were performed as described above with *K. marxianus* IMB 3 and *S. cerevisiae* D_5A with 5, 10, and 15 FPU/g glucan Fibrilase enzyme. The effect of initial buffer pH was also explored by performing SSFs with *S. cerevisiae* D_5A and sodium citrate buffer with pH 4.8.

4.6 Control of SSF pH using KOH in Bioreactor

The buffers used in the shake flask SSFs cannot maintain a constant pH and depending on the initial pH of the buffer may allow the pH to fall to levels that begin to affect the fermentative ability of the yeast. In order to maintain constant pH, two SSFs were performed in a 3-L stirred bioreactor (BIOFLO 110, New Brunswick Scientific, Edison, NJ) with automatic pH control (Figure 4.2). The bioreactor was loaded with pretreated switchgrass containing 60 g glucan, 150 mL of nutrient media, and 15 FPU/g glucan Fibrilase enzyme. 15 mL of concentrated IMB 3 yeast cells were added to provide an initial OD of 0.5. Deionized water was added to make the total mass of the SSF 1500 g. The SSFs were performed at 45 ºC while being stirred at 700 rpm. The bioreactor continuously monitored the pH of the SSF and added 2 M KOH in order to maintain the pH at 5.5 or 5.0. Samples were taken at 0, 24, 48, 72, 96, 120, 144, and 168 h. The samples were frozen for later analysis in 2 mL centrifuge tubes.

Figure 4.2 3-L BioFlo 110 Stirred Bioreactor with pH and Temperature control.

4.7 Analysis of SSF Samples by HPLC

The frozen SSF samples were thawed and centrifuged at 13,000 rpm for 12 min. The supernatant from each sample was filtered through a 0.2 µm syringe tip filter into a 1.5 mL HPLC sample vial and capped. 20 µL of each sample were analyzed by HPLC with a refractive index detector (RID) using an Aminex HPX-87H organic acid column at 60 ºC. The mobile phase was 0.01 N H_2SO_4 pumped at 0.6 mL/min for 30 min (Dowe and McMillan 2001). The samples were analyzed for cellobiose, glucose, xylose, xylitol, succinic acid, glycerol, acetic acid, and ethanol. The results of each of the three shake flask SSFs were averaged. One SSF performed with IMB4 in the initial screening experiment and one SSF with IMB 3 in the varied enzyme loading study resulted in failed fermentations. In these failed fermentations, ethanol production proceeded very slowly at the beginning of the experiment and final ethanol yield was nearly 50% less than the other SSFs with the same conditions. For these experiments, the results of only two SSFs were averaged. The average value was then corrected by subtracting the amounts of each compound found in the fermentation performed without switchgrass substrate. The experiments in the bioreactor were only performed once for each condition and are not adjusted for compounds resulting from fermentation of the nutrient media or enzyme mixture.

4.8 Statistical Analysis Methods

Statistical comparisons of mean glucan to ethanol yield by the IMB yeast was done by analysis of variance (ANOVA) and compared to the control, *S. cerevisiae* D_5A, for all sample times at 24 h and afterward with the Tukey method for comparing multiple treatments (Tukey 1949) using SAS Release 9.1 software (SAS, Cary, NC). The mean

glucan to ethanol yield produced by each IMB strain was compared to the other IMB strains using ANOVA and Tukey's method as well. ANOVA and Tukey's method were also used to compare mean glucan to ethanol yields for the varied enzyme loading studies with IMB 3 and *S. cerevisiae* D_5A as well as the mean ethanol yields from the initial buffer pH experiments with *S. cerevisiae* D_5A. Experiments were performed in triplicate unless otherwise noted and all statistical comparisons were made at a 95% confidence interval (See Appendices).

CHAPTER V

RESULTS AND DISCUSSION

5.1 Switchgrass Composition and Cellulase Activity

Native Kanlow switchgrass was subjected to extraction by ethanol and water prior to compositional analysis. A total of 10.8% of the dry material was removed as extractives, 2.2% by ethanol and 8.6% by water. The residue remaining after extraction was used to complete the compositional analysis of the native switchgrass. The total dry basis composition was 34.2% glucan, 23.3% xylan, 1.5% galactan, 2.0% arabinan, 0.5% mannan, 17.6% Klason lignin, 2.3% acid soluble lignin, 2.4% acetyl groups, 10.8% extractives, and 4.3% ash. This compositional analysis accounts for more than 99% of the dry matter in the switchgrass.

Following hydrothermolysis pretreatment, the average composition of the washed solids was 53.2% glucan, 2.6% xylan, and 33.8% lignin. Figure 5.1 shows switchgrass before and after pretreatment. The glucan and lignin content of the solids was increased by 56% and 70% respectively, while xylan content was reduced by 89%. During pretreatment, approximately 37.7% of the dry switchgrass, mainly xylan, was solubilized into the liquid fraction. Suryawati et al. (2008) found similar results for the same variety of switchgrass. The same pretreatment method increased the glucan content from 36.6% to 56.6 % and decreased xylan content from 21.0% to 2.4% (Suryawati et al. 2008).

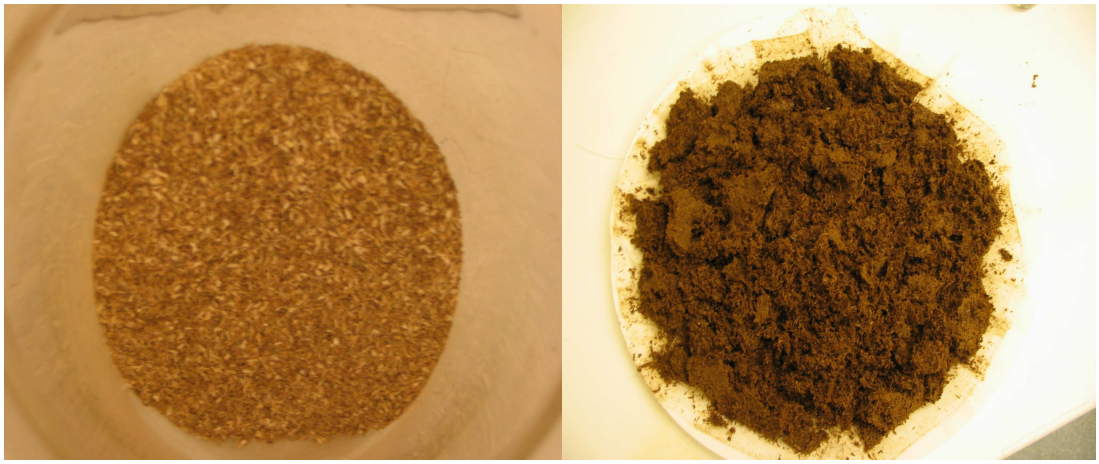

Figure 5.1 Milled Kanlow Switchgrass before (left) and following (right) Hydrothermolysis Pretreatment at 200 °C.

The cellulase activity of the Fibrilase enzyme used in these experiments was found to be approximately 65 FPU/mL on two separate occasions. Suryawati et al. (2009) used this enzyme over a year prior to these experiments and found the activity ranged from 62 to 67 FPU/mL.

5.2 Characterization of SSF of Pretreated Switchgrass by *K. marxianus* IMB strains and *S. cerevisiae* D_5A

The glucose concentration in all SSFs, shown in Figure 5.2, increased during the first 4 h indicating that hydrolysis of glucan to glucose was occurring faster than ethanol production shown in Figure 5.3. After 4 h, ethanol production occurred faster than hydrolysis, resulting in a decrease in glucose concentration. At 48 h, IMB 2 had reduced the glucose concentration to 0 g/L. IMB 1 and IMB 3 also reduced glucose concentrations below 0.3 g/L at 48 h. IMB 1 and IMB 2 maintained these glucose concentrations through 72 h, while IMB 3 maintained low glucose concentration through 96 h, indicating longer fermentation than other IMB strains. In all IMB SSFs, hydrolysis continued throughout the entire experiment. After 96 h, the glucose concentration in all IMB SSFs increased until the end of the experiment. The lowest glucose concentration after 168 h was 2.5 g/L in IMB 3 SSFs. The highest glucose concentration was 8.8 g/L in IMB 4 SSFs.

Glucose concentrations in SSFs performed with *S. cerevisiae* D_5A behaved similarly to glucose concentrations in IMB1, IMB 2, and IMB 3 through 72 h. After 72 h, however, the glucose concentration in *S. cerevisiae* D_5A SSFs continued to decrease and reached 0 g/L at 144 h. The concentration remained at 0 g/L until the end of the experiment, indicating fermentation by *S. cerevisiae* D_5A had not ceased.

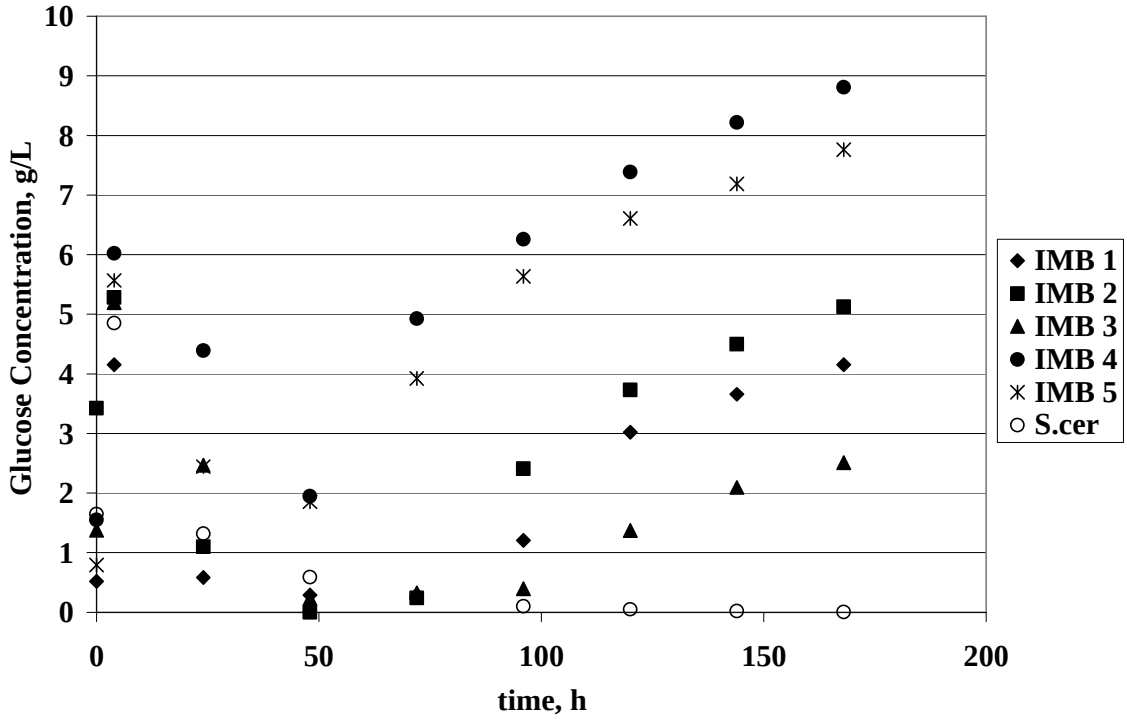

Figure 5.2 Glucose concentrations in SSFs with *K. marxianus* IMB 1, IMB 2, IMB 3, IMB 4, and IMB 5 at 45 ºC and *S. cerevisiae* D_5A at 37 ºC.

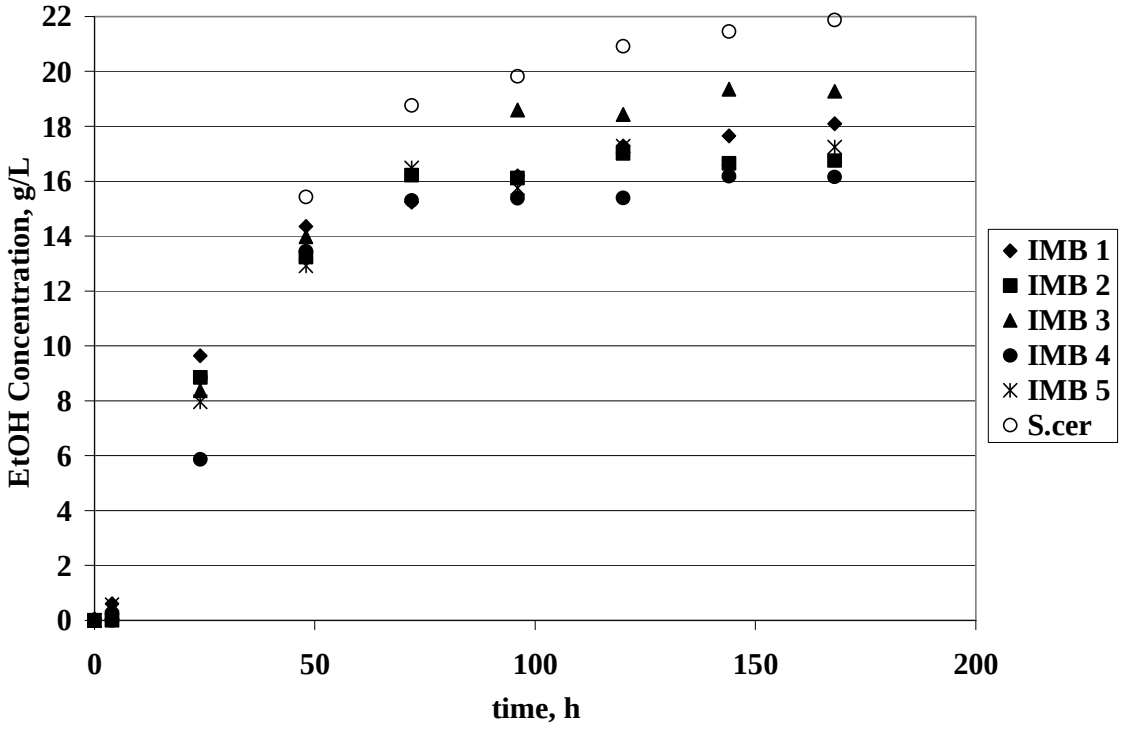

Figure 5.3 Ethanol concentrations in SSFs with *K. marxianus* IMB 1, IMB 2, IMB 3, IMB 4, and IMB 5 at 45 ºC and *S. cerevisiae* D_5A at 37 ºC.

Cellobiose concentrations in SSFs, shown in Figure 5.4, were similar to the glucose concentrations before 72 h. The cellobiose concentration increased rapidly from 0 to 4 h and then decreased as ethanol production increased. Unlike glucose in IMB SSFs, cellobiose concentrations continued to decrease after ethanol fermentation had slowed because of β-glucosidase activity in the Fibrilase enzyme mixture. All SSFs had final cellobiose concentrations of less than 0.5 g/L.

Acetic acid (Figure 5.5), glycerol (not shown), and succinic acid (not shown) were produced by all strains at concentrations below 2 g/L. IMB 1 accumulated the most acetic acid, 1.7 g/L, at 168 h. The most glycerol produced was 1.3 g/L at 96 h by IMB 3. The most succinic acid produced was 0.6 g/L by IMB 5 at 72 h.

The pH of each SSF was recorded after 168 h and the mean pH for each strain was calculated. The final mean pH values in increasing order were as follows: IMB 1, 4.56 ± 0.02; IMB 2, 4.60 ± 0.05; IMB 3, 4.64 ± 0.07; IMB 5, 4.67 ± 0.03; IMB 4, 4.71 ± 0.03 (mean of only two pH values); *S. cerevisiae* D_5A, 4.79 ± 0.06. One SSF with IMB 4 resulted in failed fermentation. The final concentration of acetic acid produced by each strain in order from highest to lowest was in the same order as increasing pH. This indicates that acetic acid production is partially responsible for the pH during SSF.

All five of the IMB strains produced more than 15 g/L of ethanol by 72 h (Figure 5.3). The highest ethanol concentration achieved by an IMB strain was 19.5 g/L by IMB 3 at 144 h. All strains, except IMB 4, produced ethanol similarly for the first 72 h. At 24 h, the concentration of ethanol produced by IMB 4, 5.9 g/L, was lower than the other four strains, which were between 8 and 10 g/L. However at 48 and 72 h, all five strains produced similar amounts of ethanol. Fermentation by IMB 1, IMB 2, IMB 4, and IMB 5

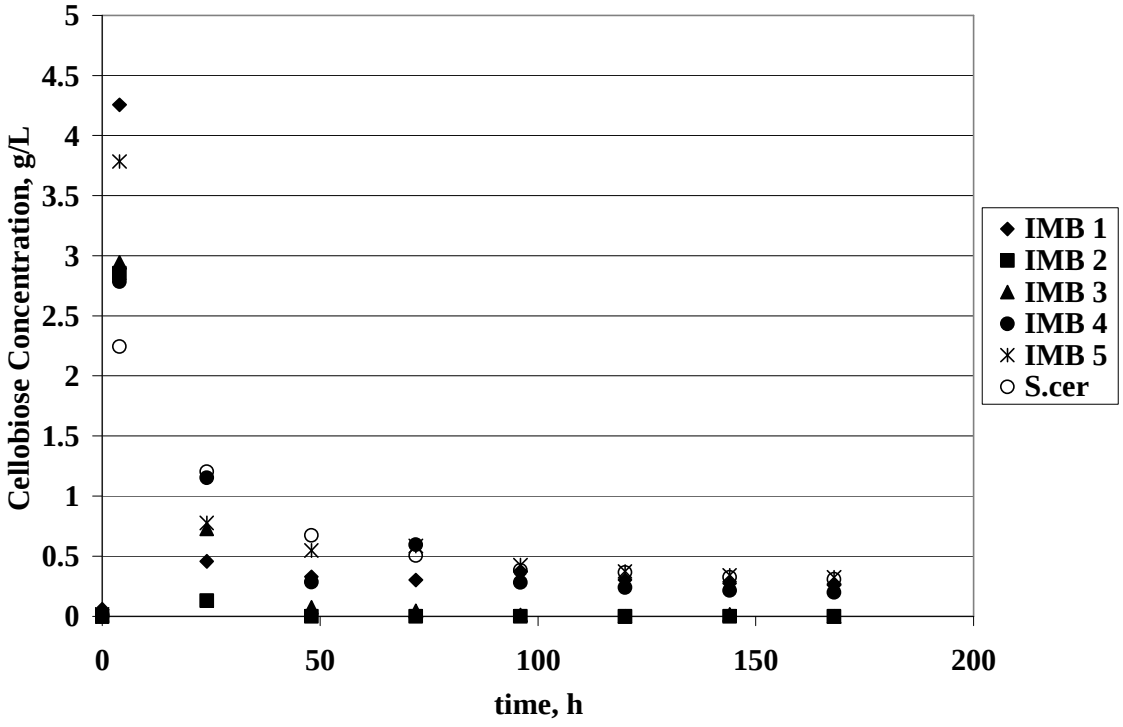

Figure 5.4 Cellobiose concentrations in SSFs with *K. marxianus* IMB 1, IMB 2, IMB 3, IMB 4, and IMB 5 at 45 ºC and *S. cerevisiae* D_5A at 37 ºC.

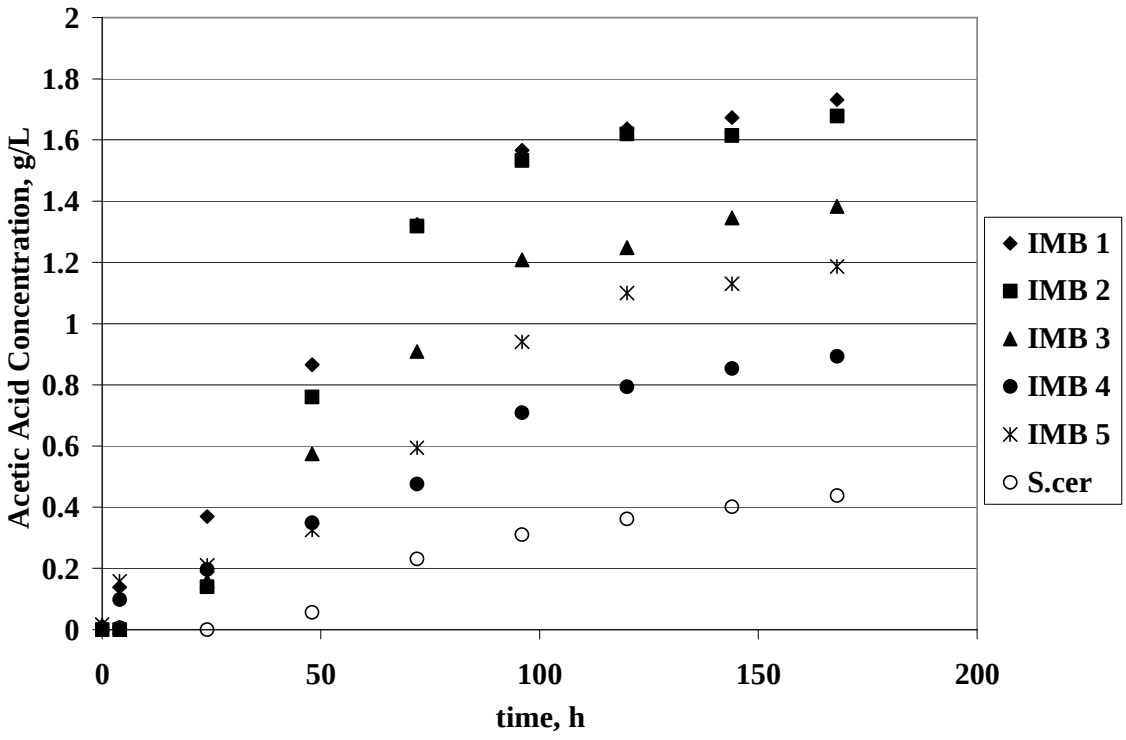

Figure 5.5 Acetic Acid concentrations in SSFs with *K. marxianus* IMB 1, IMB 2, IMB 3, IMB 4, and IMB 5 at 45 ºC and *S. cerevisiae* D_5A at 37 ºC.

slowed after 72 h. This can also be seen in Figure 5.2 as the glucose concentrations begin to increase after 72 h, indicating glucan hydrolysis was occurring faster than fermentation. IMB 3 fermentation remained faster than hydrolysis until 96 h, as is evidenced by the glucose concentration remaining near 0 g/L. Ethanol production by *S. cerevisiae* proceeded similarly to the IMB strains through 72 h, after which, ethanol production continued until the end of the experiment, reaching a maximum concentration of 21.9 g/L at 168 h.

The percentage of maximum theoretical ethanol produced from glucan was calculated using the following equation:

$$\%\ Theoretical\ \ Maximum\ \ Ethanol = \frac{[EtOHt] - [EtOHo]}{0.511\ x\ (f[Biomass]\ x\ 1.11)}\ x\ 100\%$$

$[EtOH_t]$ – ethanol concentration at time t (g/L), $[EtOH_0]$ – ethanol concentration at time 0 (g/L), 0.511 – mass conversion factor of glucose to ethanol (g/g), f – fraction of glucan in dry solids (g/g), [Biomass] – initial concentration of solids (g/L), and 1.11 – mass conversion factor of glucan hydrolysis to glucose (g/g)

No difference was seen in theoretical ethanol yields of the five IMB strains at any sample time during the experiment ($p>0.05$). At 72 h, glucan to ethanol yields by all IMB yeasts had achieved between 60 and 70% conversion (Figure 5.6 and Table 5.1). IMB 3 achieved 77% conversion by 96 h, but conversion to ethanol by IMB 1, IMB 2, IMB 4, and IMB 5 remained below 70%. By 168 h, IMB 1 and IMB 3 achieved 75% and 80% conversion, respectively. *S. cerevisiae* D_5A produced 83% of maximum theoretical ethanol at 96 h and slightly greater than 90 % at 168 h. At 96 h and afterward, ethanol yield by *S. cerevisiae* D_5A was greater than ethanol yields by IMB 1, IMB 2, IMB 4 and IMB 5 ($p<0.05$), but ethanol yields by IMB 3 and *S. cerevisiae* D_5A were not

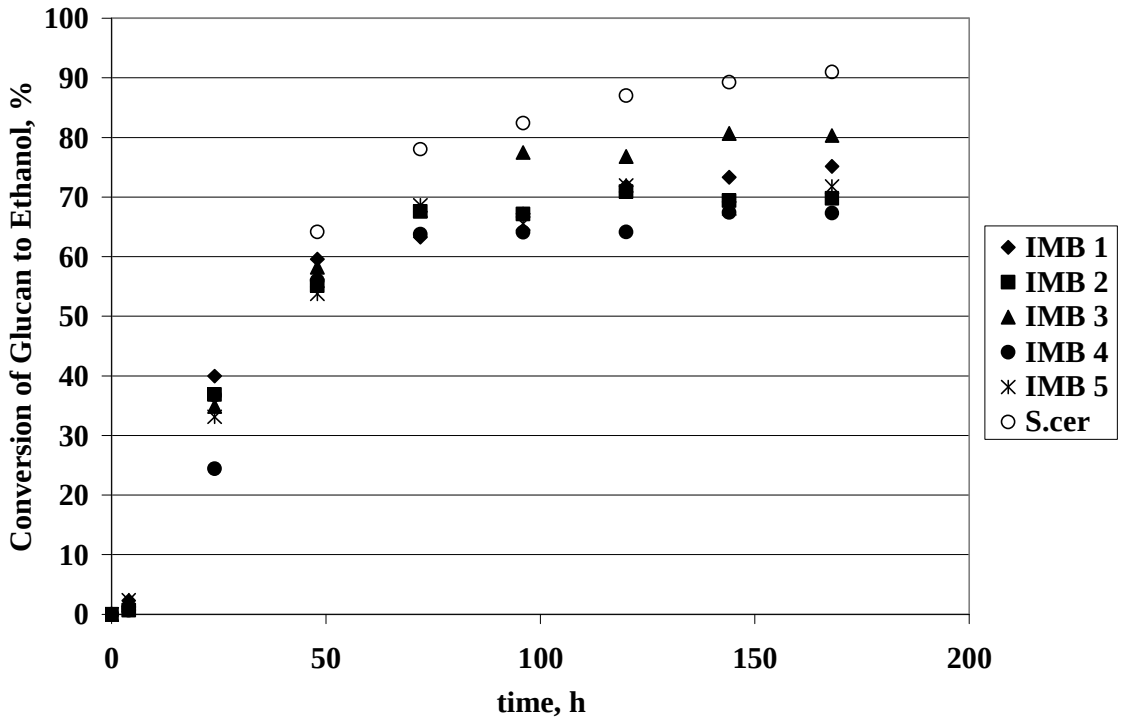

Figure 5.6 Percent of maximum theoretical conversion of glucan to ethanol in SSFs with *K. marxianus* IMB 1, IMB 2, IMB 3, IMB 4, and IMB 5 at 45 ºC and *S. cerevisiae* D_5A at 37 ºC.

Table 5.1 Mean percent of maximum theoretical conversion of glucan to ethanol in SSFs with *K. marxianus* IMB 1, IMB 2, IMB 3, IMB 4, and IMB 5 at 45 ºC and *S. cerevisiae* D_5A at 37 ºC.

Strain	72 h	96 h	120 h	144 h	168 h
K. marxianus IMB 1	63.3(5.3)[b]	67.2(4.2)[b]	71.8(7.0)[b]	73.3(4.5)[b]	75.2(1.2)[b]
K. marxianus IMB 2	67.6(2.1)[a,b]	67.1(8.4)[b]	70.9(1.5)[b]	69.4(1.9)[b]	69.8(1.2)[b]
K. marxianus IMB 3	67.7(3.3)[a,b]	77.5(4.6)[a,b]	76.8(5.5)[a,b]	80.7(1.2)[a,b]	80.3(1.1)[a,b]
K. marxianus IMB 4	63.8(4.0)[b]	64.1(8.6)[b]	64.1(1.7)[b]	67.4(6.9)[b]	67.3(6.4)[b]
K. marxianus IMB 5	68.6(8.2)[a,b]	65.6(3.9)[b]	72.0(9.3)[b]	68.1(9.1)[b]	71.8(9.5)[b]
S. cerevisiae D_5A	79.5(1.0)[a]	86.3(0.8)[a]	89.2(1.7)[a]	91.7(1.5)[a]	92.3(3.6)[a]

The number in parenthesis is the standard deviation of the mean value of glucan to ethanol yield.

[a] indicates the mean ethanol yield for IMB strains is similar to that of *S. cerevisiae* D_5A based on a 95% confidence interval.

significantly different at any sample time during the experiment ($p>0.05$). IMB 3 was chosen for use in subsequent experiments because it consumed glucose 24 h longer than other IMB strains and compared best to the *S. cerevisiae* D_5A control in terms of ethanol yield.

Previous research has found similar ethanol production results at elevated temperatures to those of the *K. marxianus* strains used in this study. Lark et al. (1997) performed SSF on recycled paper sludge using *K. marxianus* at 38 ºC. After 72 h, 72% of cellulose was converted to ethanol. Using *K. marxianus* CECT 10875 in SSFs of lignocellulosic material at 42 ºC, Ballesteros et al. (2004) achieved glucan to ethanol conversions ranging from 50% to 72%. Using *K. marxianus* IMB 3 at 45 ºC in SSFs of pretreated straw, Barron et al. (1997) converted between 75% and 86% of cellulose to ethanol. In Suryawati et al. (2008), SSFs of switchgrass with similar conditions using IMB 4 resulted in 16.6 g/L of ethanol at 72 h and a final theoretical ethanol yield of 79%. Results from this experiment using IMB 4 were slightly lower achieving 15.3 g/L of ethanol at 72 h and a final theoretical yield of 70%.

The results of this work show that SSF of switchgrass by *S. cerevisiae* D_5A at 37 ºC outperformed all of the *K. marxianus* IMB strains, except IMB 3, at 45 ºC in terms of ethanol yield. However, when the initial pH of the SSF buffer was 4.8 instead of 5.5, the final ethanol yield by *S. cerevisiae* D_5A was only 79% (Suryawati et al. 2008), compared to 92% in this study. Suryawati et al. (2008) also found that increasing the initial buffer pH from 4.8 to 5.5 for SSFs with IMB 4 at 45 ºC increased final ethanol yield from 69% to 79%, the same final yield as was produced by *S. cerevisiae* D_5A with buffer pH 4.8. However, IMB 4 produced this yield 72 h earlier than *S. cerevisiae* D_5A.

5.3 Effect of Initial Buffer pH on SSF of Pretreated Switchgrass by *S. cerevisiae* D_5A

This experiment was performed because Suryawati et al. (2008) found that SSF of switchgrass with *S. cerevisiae* D_5A at 37 ºC with initial buffer pH of 4.8 resulted in a final ethanol yield of 79%, but the same experiment done previously in this work with initial buffer pH of 5.5 yielded 92%. It was found that lowering the pH of the buffer used in shake flask SSFs with *S. cerevisiae* D_5A from 5.5 to 4.8 affected glucose consumption and ethanol yield.

In SSFs with buffer pH 5.5, glucose concentration increased rapidly through the first 4 h, as shown in Figure 5.7, followed by a continuous decrease as glucose was consumed by the yeast. The final glucose concentration was 0.1 g/L. This was consistent with experiments previously discussed in this work. Glucose concentration in SSFs with buffer pH 4.8 also increased rapidly through the first 4 h followed by a decrease in concentration as the yeast began consuming glucose. However, after 72 h, glucose concentrations began increasing and reached 3.7 g/L at the end of the experiment, indicating fermentation by the yeast had slowed compared to glucan hydrolysis. This result was not seen in any SSFs performed with *S. cerevisiae* D_5A and initial buffer pH of 5.5. *S. cerevisiae* D_5A with buffer pH 4.8 in Suryawati et al. (2008) maintained glucose concentrations below 0.5 g/L at the end of the experiment.

Production of acetic acid and ethanol was also affected by the difference in buffer pH. Acetic acid concentrations, shown in Figure 5.8, with both buffers increased until the end of the experiment, and SSFs with buffer pH 5.5 produced more acetic acid, 0.54

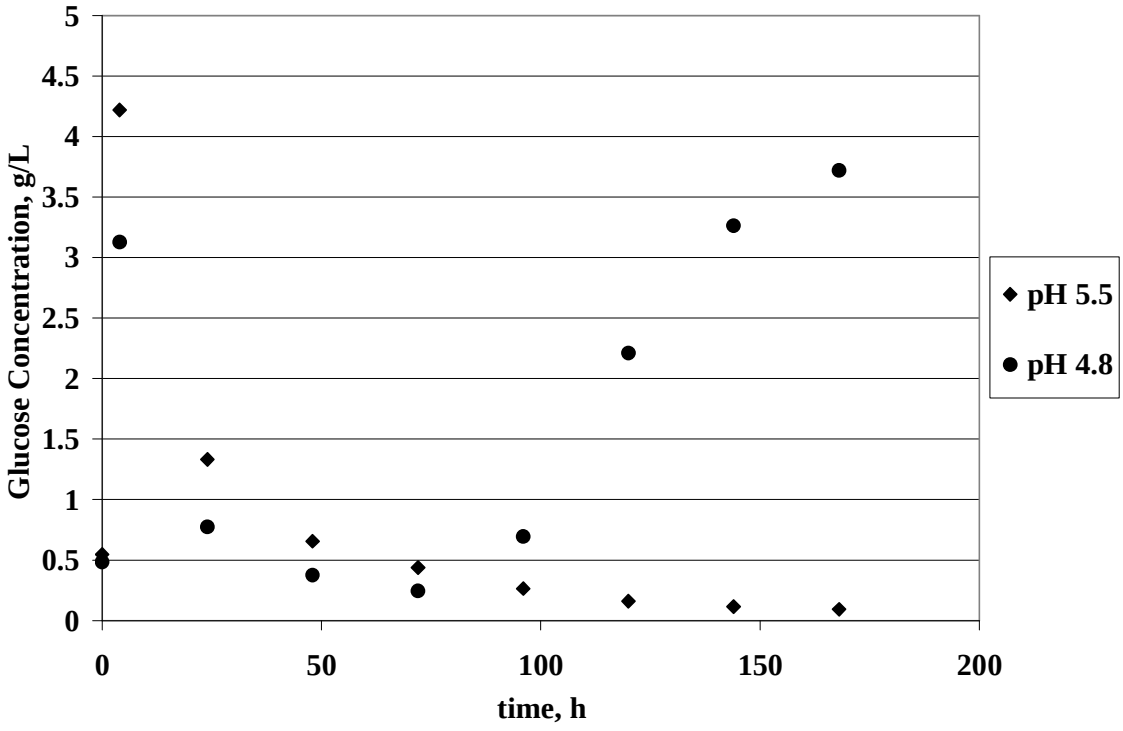

Figure 5.7 Glucose concentrations in SSFs with *S. cerevisiae* D_5A at 37 ºC with buffer pH 4.8 and 5.5.

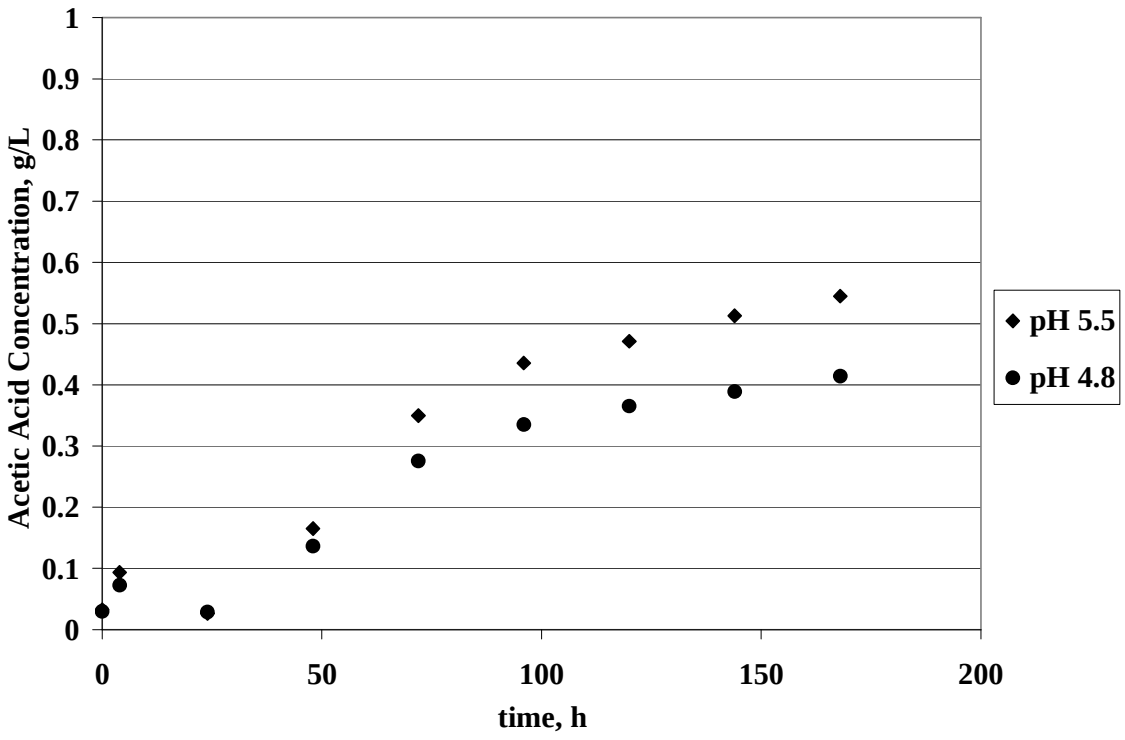

Figure 5.8 Acetic Acid concentrations in SSFs with *S. cerevisiae* D_5A at 37 ºC with buffer pH 4.8 and 5.5.

g/L, than SSFs with buffer pH 4.8, 0.41 g/L. However, glucan to ethanol yield was higher for pH 5.5 SSFs in every sample after 48 h ($p<0.05$). This indicates that the total quantity of acetic acid present does not determine its inhibitory effects. Instead, the quantity of undissociated acetic acid present due to pH determines inhibitory effects. After 96 h, pH 4.8 SSFs converted 76% of glucan to ethanol while pH 5.5 SSFs converted 86% (Figure 5.9). By the end of the experiment, pH 4.8 SSF glucan to ethanol conversion had increased slightly to 78%, but pH 5.5 SSF conversion had increased to 92%. Similar to these results, *S. cerevisiae* D_5A with pH 4.8 buffer in Suryawati et al. (2008) yielded 68% at 96 h and 79% at 168 h.

The average final pH for SSFs with buffer pH of 4.8 and 5.5 was 4.23 and 4.69, respectively. The final pH of SSFs with both buffers was below 4.74, the pKa of acetic acid (Freese et al. 1973). The concentration of undissociated acetic acid increases as pH decreases, and the inhibitory effects on yeast are increased (Narendranath et al. 2001). Thus, the results are expected that yeast performance is diminished as the pH of the SSF is decreased.

5.4 SSF of Pretreated Switchgrass by *K. marxianus* IMB 3 at 45 ºC in pH-controlled Bioreactor

As discussed previously, pH has a significant effect on the performance of the yeast, particularly when the pH drops below the pKa of acetic acid. Buffers cannot maintain constant pH throughout an SSF. Despite the initial pH of the buffer, the performance of the yeast may be diminished as pH decreases. The purpose of this experiment was to explore the effects of maintaining the pH of SSFs with *K. marxianus* IMB 3 at 5.0 and 5.5 using a stirred bioreactor with pH control.

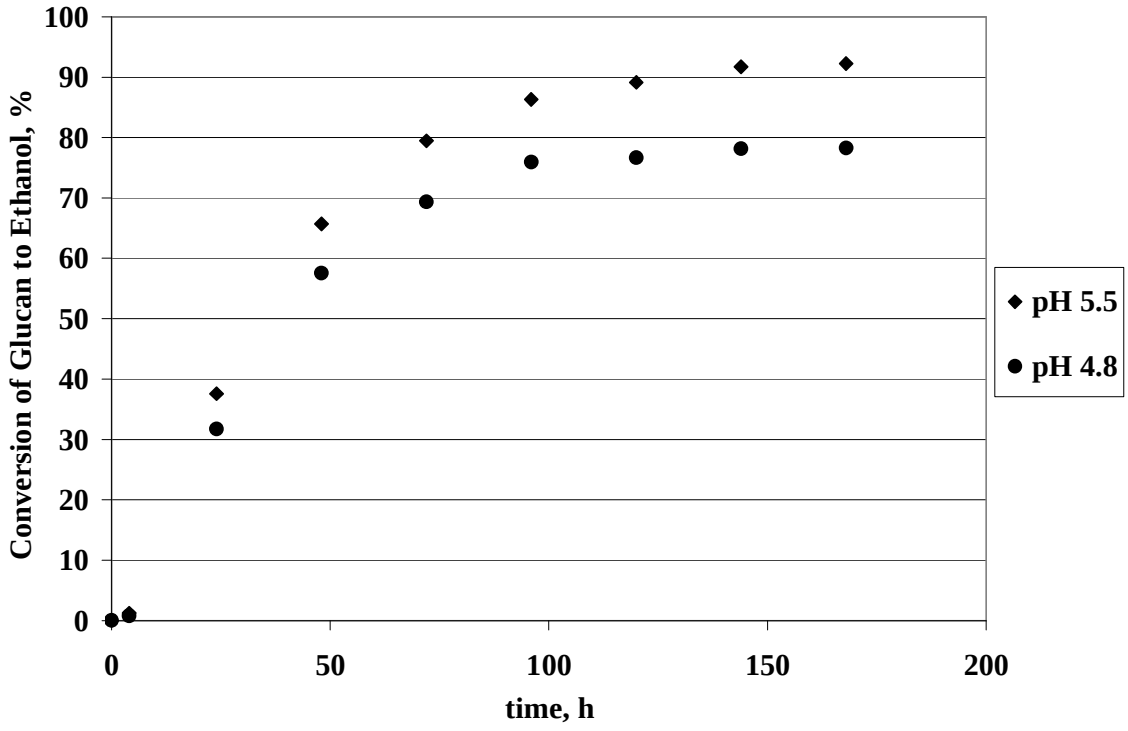

Figure 5.9 Percent of maximum theoretical conversion of glucan to ethanol for *S. cerevisiae* D_5A SSFs at 37 ºC with buffer pH of 4.8 or 5.5.

Controlling the pH at 5.5 during SSF by IMB 3 resulted in a delayed start of fermentation compared to previous shake flask experiments. Fermentation did not begin until 24 h into the experiment. Without the yeast consuming glucose, the glucose concentration reached 16.1 g/L at 24 h in Figure 5.10. This was the highest glucose concentration seen in the experiments performed for this work. Once the yeast began fermenting, the glucose concentration was reduced to 0.5 g/L by 72 h. The glucose concentration remained below 0.5 g/L until 120 h. The final glucose concentration was 2.0 g/L. Fermentation in the SSF controlled at pH 5.0 started earlier and kept the glucose concentration at 24 h, 6.8 g/L, lower than the SSF at pH 5.5, 16.1 g/L. At 48 h, the glucose concentration was reduced to 0.4 g/L and remained below 0.4 g/L through 96 h. After 96 h, glucose concentration began increasing and reached 5.1 g/L at the end of the experiment.

Acetic acid concentrations increased throughout the experiment for both pH control levels reaching 0.74 g/L at pH 5.0 and 0.88 g/L at pH 5.5 after 168 h (Figure 5.11). In shake flask SSFs with IMB 3, the final acetic acid concentration reached 1.38 g/L, indicating that acetic acid production is reduced by maintaining the pH at these levels. The concentration of ethanol in the SSF controlled at pH 5.0 increased from the beginning of the experiment until 120 h (Figure 5.12). At 120 h, the concentration reached 18.5 g/L or 83.5% of theoretical maximum ethanol yield. After 120 h, ethanol concentration remained constant until the end of the experiment. In the pH 5.5 SSF, ethanol fermentation began after 24 h and continued to increase until the end of the experiment. At 168 h, the ethanol concentration was 17.6 g/L or 77.5% of theoretical maximum ethanol yield. Shake flask SSFs with IMB 3 and initial buffer pH 5.5 resulted

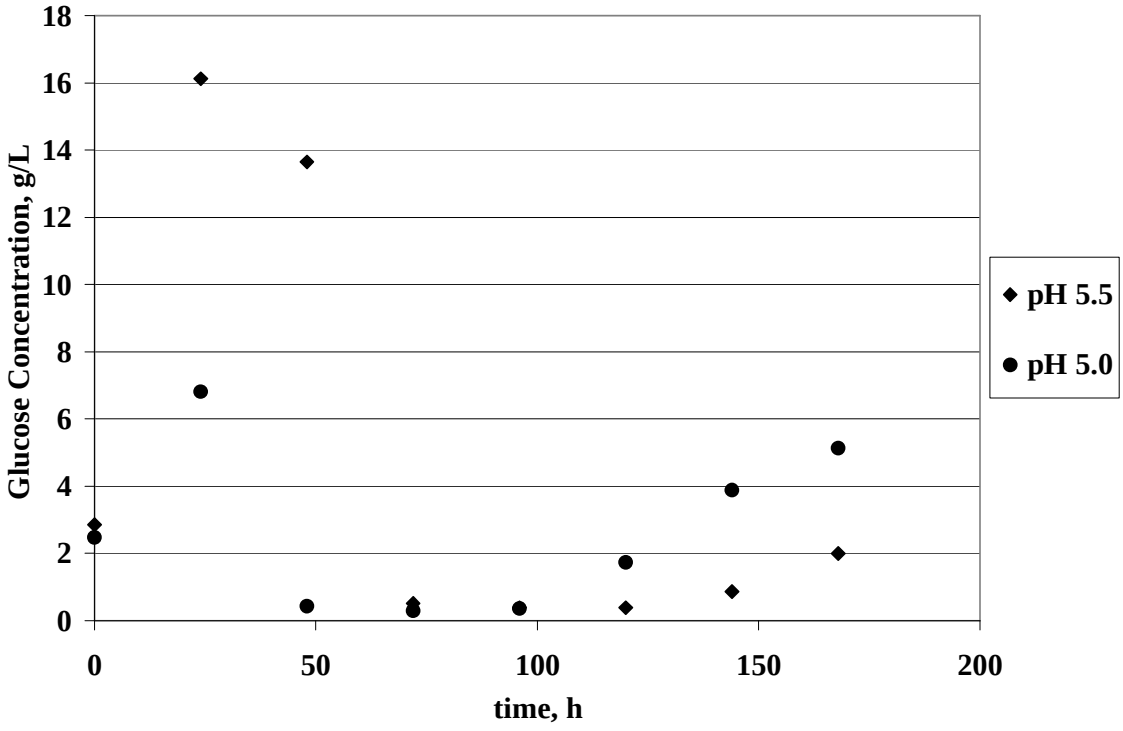

Figure 5.10 Glucose concentrations in SSFs with *K. marxianus* IMB 3 at 45 ºC in bioreactor controlled at pH 5.0 and 5.5.

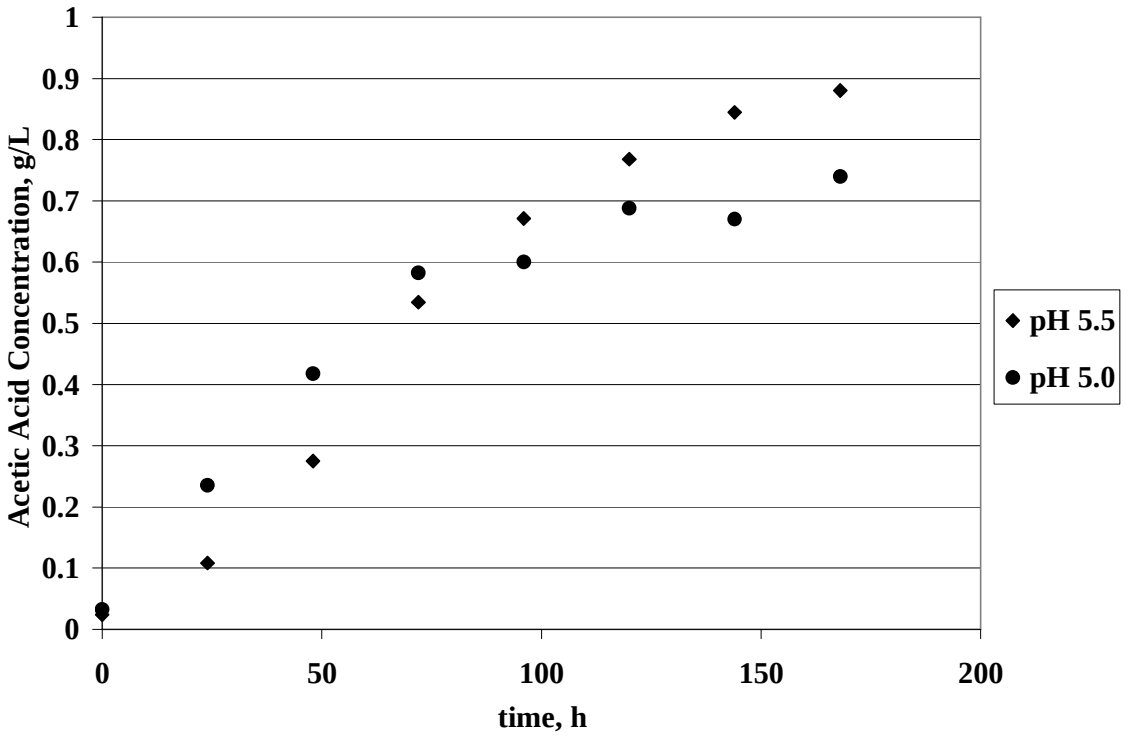

Figure 5.11 Acetic Acid concentrations in SSFs with *K. marxianus* IMB 3 at 45 ºC in bioreactor controlled at pH 5.0 and 5.5.

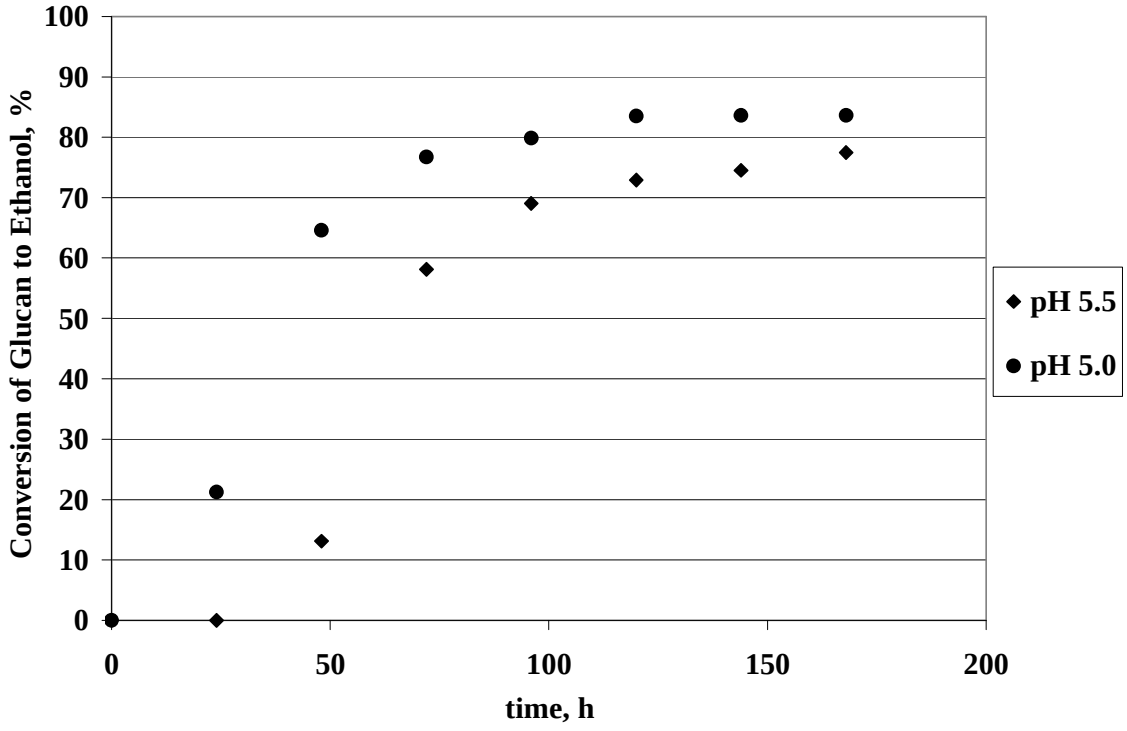

Figure 5.12 Percent of maximum theoretical conversion of glucan to ethanol for *K. marxianus* IMB 3 SSFs at 45 ºC in bioreactor controlled at pH of 5.0 and 5.5.

in a maximum ethanol yield of 78% reached after 96 h. These results indicate that maintaining the pH of the SSF at 5.0 increases the ethanol yield by extending the fermentation time of IMB 3 past 96 h, but maintaining the pH at 5.5 may cause a longer lag period at the beginning of the fermentation and does not increase overall ethanol yield.

5.5 Effect of Reduced Cellulase Enzyme Loading on SSF of Pretreated Switchgrass by *K. marxianus* IMB 3 and *S. cerevisiae* D_5A

Reducing the cellulase enzyme loading from 15 FPU/g glucan to 5 or 10 FPU/g glucan in SSFs with *K. marxianus* IMB 3 at 45º C and *S. cerevisiae* D_5A at 37 ºC resulted in reduced glucan hydrolysis and lowered ethanol production. The difference in initial hydrolysis rates can be seen after 4 h in Figures 5.13 and 5.14. IMB 3 SSFs with 15 FPU/g glucan had the highest 4 h glucose concentration, 6.5 g/L, and IMB 3 SSFs with 5 and 10 FPU/g glucan had lower glucose concentrations of 0.7 and 3.7 g/L, respectively. Glucose was rapidly consumed by IMB 3 yeast in all enzyme treatment levels resulting in decreased concentrations at 24 and 48 h. IMB 3 fermentation slowed after 72 h and glucose concentrations increased until the end of the experiment. The highest final glucose concentration, 15.0 g/L, was reached by IMB 3 SSFs with 10 FPU/g glucan at 168 h. Ethanol production, shown in Figure 5.15, decreased with lowered enzyme loadings as well. Ethanol yields at 72 h and afterwards by IMB 3 SSFs with lowered enzyme loadings were less than the control enzyme loading level of 15 FPU/g glucan ($p<0.05$). IMB 3 SSFs with 5 and 10 FPU/g glucan converted only 41% and 62% of glucan to ethanol, respectively, while SSFs with 15 FPU/g glucan converted 78%. The results with 15 FPU/g glucan are similar to those seen by IMB 3 in the previous

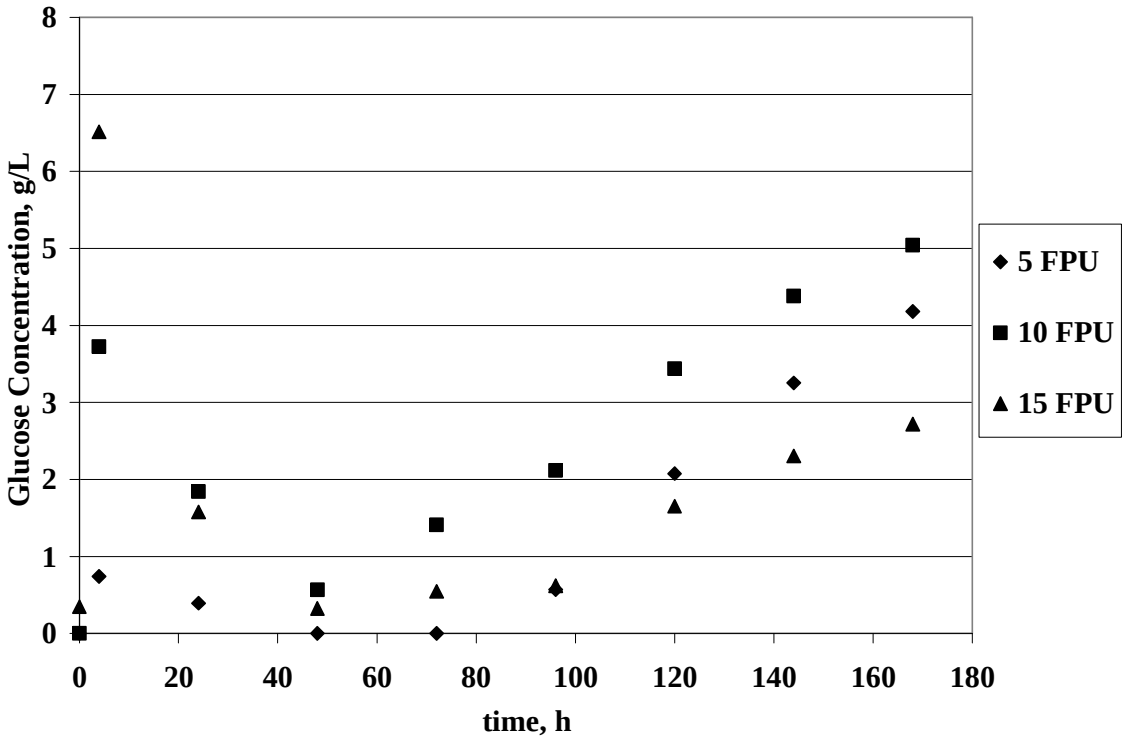

Figure 5.13 Glucose concentrations in *K. marxianus* IMB 3 SSFs with 5, 10, and 15 FPU/g glucan at 45 ºC.

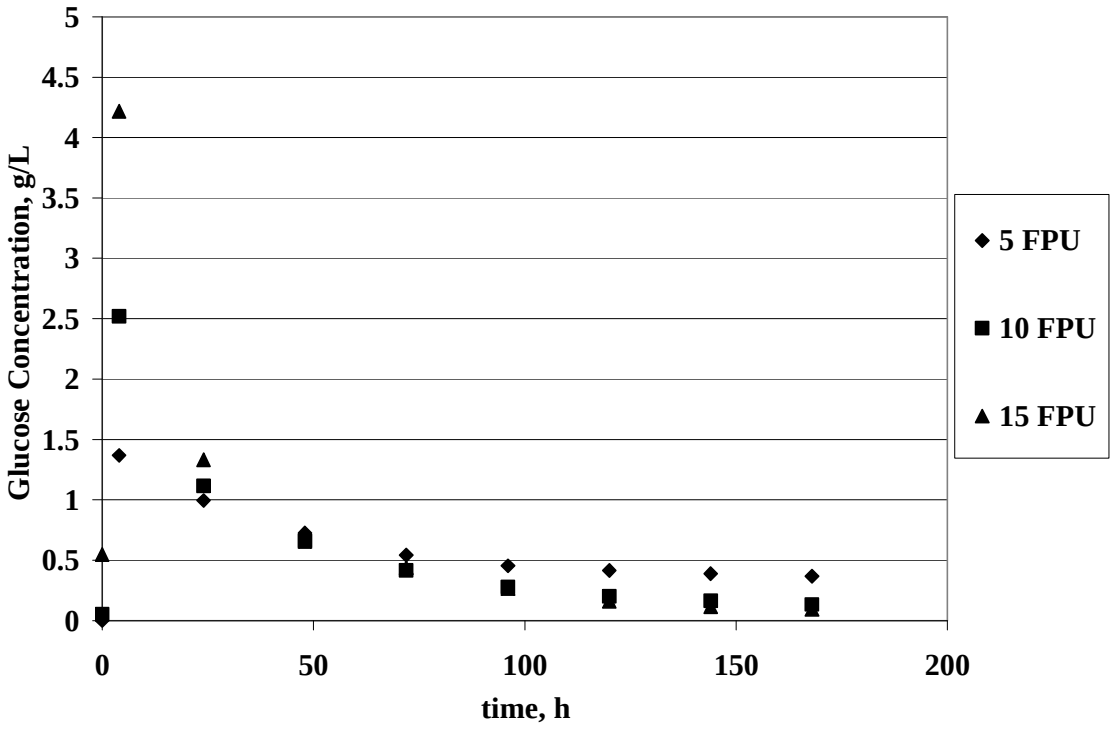

Figure 5.14 Glucose concentrations in *S. cerevisiae* D_5A SSFs with 5, 10, and 15 FPU/g glucan at 37 ºC.

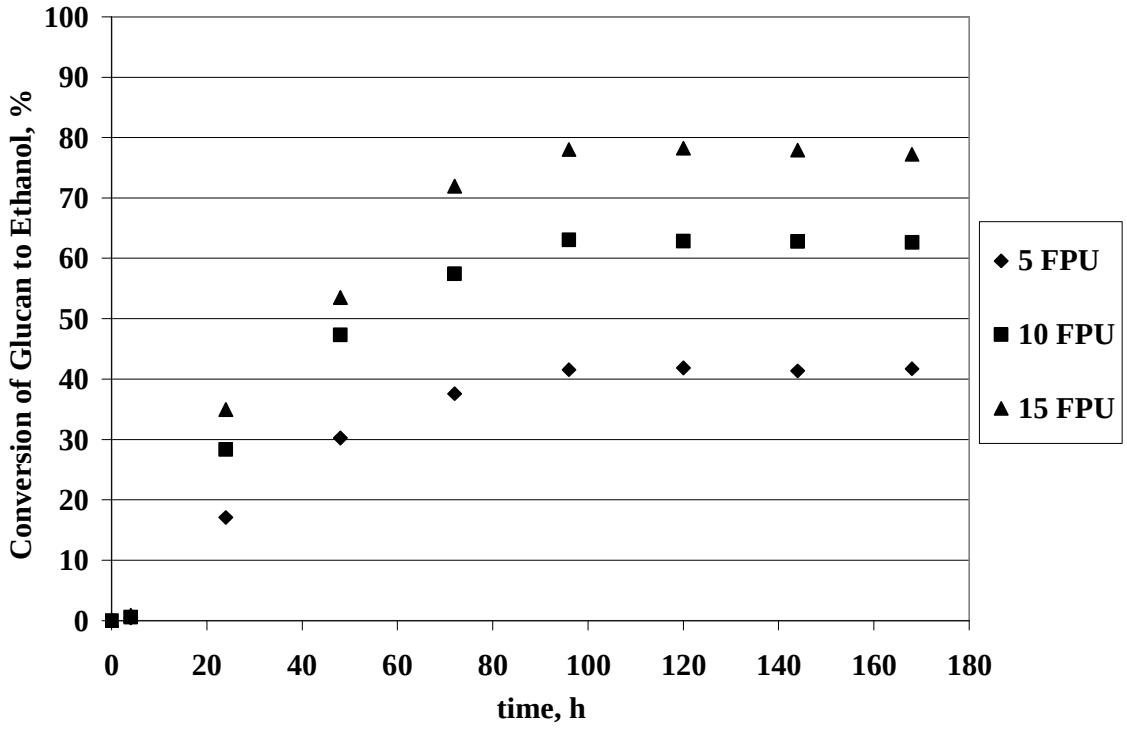

Figure 5.15 Percent of maximum theoretical conversion of glucan to ethanol for *K. marxianus* IMB 3 SSFs with 5, 10, 15 FPU/g glucan at 45 ºC.

experiment of this chapter. The maximum ethanol concentrations reached by the IMB 3 SSFs were 10.0, 15.0, and 18.7 g/L for enzyme loadings of 5, 10, and 15 FPU/g glucan, respectively.

SSFs with *S. cerevisiae* D_5A at 37 ºC and lowered enzyme also resulted in lowered glucan to ethanol yields. Glucose concentrations, shown in Figure 5.14, after 4 h were 1.4, 2.5, and 4.3 g/L for enzyme loadings of 5, 10, and 15 FPU/g glucan, respectively. The glucose concentrations in SSFs with 10 and 15 FPU/g glucan after 4 h at 37 ºC were lower than the glucose concentrations seen at 45 ºC with IMB 3. After 4 h, glucose concentrations in all *S. cerevisiae* D_5A SSFs decreased during the remainder of the experiment. Final glucose concentrations for all three enzyme loadings were less than 0.5 g/L. Unlike SSFs with IMB 3, *S. cerevisiae* D_5A continued fermentation for the entire duration of the experiment, and ethanol concentrations for all three enzyme loadings increased until the experiment ended. Similar to IMB 3 SSFs, decreased enzyme loadings resulted in lower ethanol production after 48 h ($p<0.05$). However, *S. cerevisiae* D_5A produced higher ethanol yields compared to IMB 3. After 96 h, the ethanol yields for *S. cerevisiae* D_5A SSFs with 5, 10, and 15 FPU/g glucan were 46%, 74%, and 86% theoretical, respectively. The maximum ethanol concentrations reached by SSFs with *S. cerevisiae* D_5A and 5, 10, and 15 FPU/g glucan were 13.0, 19.4, and 20.9 g/L, respectively. The corresponding glucan to ethanol yields were 57%, 86%, and 92% (Figure 5.16). The results with 15 FPU/g glucan are similar to those seen previously.

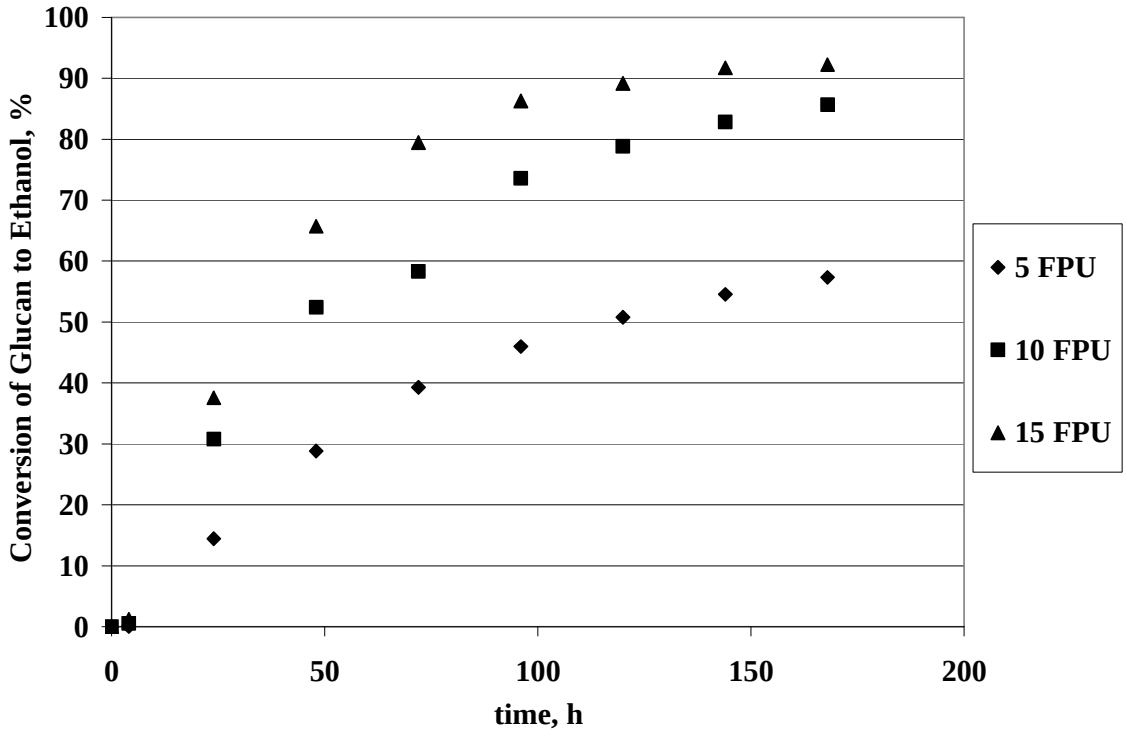

Figure 5.16 Percent of maximum theoretical conversion of glucan to ethanol for *S. cerevisiae* D_5A SSFs with 5, 10, and 15 FPU/g glucan at 37 ºC.

CHAPTER VI

CONCLUSIONS

Pretreatment of switchgrass by hydrothermolysis at 200 ºC for 10 min increased glucan content from 34% to 53% and decreased xylan content from 23% to 2.6%. SSF of this pretreated switchgrass allowed 92% of glucan to be converted to ethanol by *S. cerevisiae* D_5A at 37 ºC. On this substrate, five IMB strains of *K. marxianus* were screened for ethanol production characteristics by SSF at 45 ºC and no significant difference was seen in ethanol yields produced by the five strains ($p>0.05$). After 96 h, the IMB strains produced between 64% and 78% of maximum theoretical ethanol. Through the first 72 h, ethanol production by the five strains was similar, but IMB 1, IMB 2, IMB 4, and IMB 5 fermentation slowed after 72 h while IMB 3 continued fermentation until 96 h. The results of this work show that the IMB strains of *K. marxianus* have potential to be used for ethanol production in SSF processes at 45 ºC. However, further improvements in ethanol yield are needed because the control SSFs with *S. cerevisiae* D_5A at 37 ºC produced higher ethanol yields than all of the IMB strains except IMB 3 ($p<0.05$).

A buffer with pH 5.5 should be used in SSFs of pretreated switchgrass because reducing the pH of the buffer used in SSFs with *S. cerevisiae* D_5A at 37 ºC from 5.5 to 4.8 resulted in lower ethanol yields. After 96 h, fermentation slowed in SSFs with buffer pH 4.8. The ethanol yield was 76% at 96 h and only increased to 78% by the end of the

experiment. SSFs with buffer pH 5.5 continued fermentation during the entire experiment reaching an ethanol yield of 86% at 96 h and 92% at the end of the experiment. The effects of undissociated acetic acid should be considered when using NREL LAP-008 (Dowe and McMillan 2001) for SSF experiments which recommends using a buffer with pH 4.8.

Maintaining the pH of SSFs at 45 ºC with *K. marxianus* IMB 3 at 5.0 by automatic addition of KOH in a bioreactor extended the fermentation time past the 96 h seen in previous shake flask experiments without continuous pH control and resulted in an increase in ethanol yield from 78% to 83%. The production of acetic acid, a fermentation inhibitor, was also reduced from 1.38 g/L in buffered shake flask SSFs to 0.74 g/L in the bioreactor.

The reasons the IMB strains did not maintain fermentation activity throughout the duration of these experiments is not clear. pH control at 5.0 appears to extend fermentation time somewhat, but not comparable to the fermentation ability of the *S. cerevisiae* D_5A control SSFs at 37 ºC. The cause is unlikely ethanol inhibition because the IMB strains have shown the ability to maintain cell growth and ethanol production in solutions containing more than 75 g/L ethanol (Banat and Marchant 1995), more than three times the concentration produced in these experiments. A nutrient deficiency stemming from the prescribed nutrient media used in these experiments may explain the shortened fermentation time. Increasing the number of cells added at the beginning of the SSF may improve ethanol yield and increase fermentation time due to the increased number of viable cells.

The cellulase enzyme loading cannot be reduced from 15 FPU/g glucan without significant reductions in ethanol yields produced during SSF with *K. marxianus* IMB 3 or *S. cerevisiae* D_5A. Reducing the cellulase enzyme loading in SSFs with *K. marxianus* IMB 3 at 45 ºC from 15 FPU/g glucan to 5 and 10 FPU/g glucan reduced ethanol yield from 78% to 41% and 62%, respectively. Ethanol yields with IMB 3 were lower than the corresponding yields with 5, 10, and 15 FPU/g glucan and *S. cerevisiae* D_5A at 37 ºC which were 57, 86, and 92%, respectively.

CHAPTER VII

FUTURE WORK

Further work is needed to improve cellulose to ethanol yields and lengthen the fermentation time of the *K. marxianus* IMB strains. Increasing the cell density added at the beginning of the SSF should be investigated to determine if an increased number of viable cells have an effect on ethanol yield. In conjunction with increasing cell density, an investigation should be done on the effects that the composition of the nutrient media has on the fermentation characteristics of IMB yeast. The nutrient media used in these experiments may be deficient in or lacking altogether certain minerals or nutrients that would allow the yeast to withstand higher temperatures for longer periods of time. Suryawati et al. (2008) addressed this issue by tripling the concentration of the nutrient media in SSFs with IMB 4. However, this resulted in a decrease in ethanol yield to less than 60% conversion and no extension of fermentation time. Therefore, a more in depth study of the media composition is required. In future studies, care should be taken to ensure that cells are not exposed to rapid temperature increases. Piper (1993) showed that cells are preconditioned to thermotolerance by mild temperature increases. IMB yeasts should also be used in a new study with advanced cellulase enzyme systems that have been developed specifically for ethanol production from cellulosic biomass. Utilizing more advanced enzyme systems may improve the overall efficiency of the process leading to higher ethanol yields and allow the use of less enzyme, potentially decreasing production costs.

REFERENCES

Adler E. 1977. Lignin chemistry - past, present and future. Wood Science and Technology 11(3):169-218.

Allen SG, Kam LC, Zemann AJ, Antal MJ. 1996. Fractionation of sugar cane with hot, compressed, liquid water. Industrial & Engineering Chemistry Research 35(8):2709-2715.

Allen SG, Schulman D, Lichwa J, Antal MJ, Laser M, Lynd LR. 2001. A comparison between hot liquid water and steam fractionation of corn fiber. Industrial & Engineering Chemistry Research 40(13):2934-2941.

Anderson DW, Coleman DC. 1985. The dynamics of organic-matter in grassland soils. Journal of Soil and Water Conservation 40(2):211-216.

Anonymous. 2009. Crude Oil Price History. New York: http://www.nyse.tv/crude-oil-price-history.htm.

Antal MJ. 1996. Water: A traditional solvent pregnant with new applications. In: White HJ, editor. Proceedings of the 12th International Conference on the Properties of Water and Steam. New York: Begell House. p 24-32.

Bajpai P, Margaritis A. 1987. The effect of temperature and pH on ethanol production by free and immobilized cells of *Kluyveromyces marxianus* grown on Jerusalem artichoke extract. Biotechnology and Bioengineering 30(2):306-313

Ballesteros I, Ballesteros M, Cabanas A, Carrasco J, Martin C, Negro MJ, Saez F, Saez R. 1991. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Applied Biochemistry and Biotechnology 28-29:307-315.

Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I. 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochemistry 39(12):1843-1848.

Banat IM, Marchant R. 1995. Characterization and potential industrial applications of 5 novel, thermotolerant, fermentative, yeast strains. World Journal of Microbiology & Biotechnology 11(3):304-306.

Banat IM, Nigam P, Marchant R. 1992. Isolation of thermotolerant, fermentative yeasts growing at 52 degrees C and producing ethanol at 45 degrees C and 50 degrees C. World Journal of Microbiology & Biotechnology 8(3):259-263.

Banat IM, Nigam P, Singh D, Marchant R, McHale AP. 1998. Ethanol production at elevated temperatures and alcohol concentrations: Part I - Yeasts in general. World Journal of Microbiology & Biotechnology 14(6):809-821.

Barron N, Marchant R, McHale L, McHale AP. 1994. Growth of a thermotolerant ethanol producing strain of *Kluyveromyces marxianus* on cellobiose containing media. Biotechnology Letters 16(6):625-630.

Barron N, Mulholland H, Boyle M, McHale AP. 1997. Ethanol production by *Kluyveromyces marxianus* IMB3 during growth on straw-supplemented whiskey distillery spent wash at 45 degrees C. Bioprocess Engineering 17(6):383-386.

Bobleter O. 1994. Hydrothermal degradation of polymers derived from plants. Progress in Polymer Science 19(5):797-841.

Bobleter O, Concin R. 1979. Degradation of poplar lignin by hydrothermal treatment. Cellulose Chemistry and Technology 13:583-593.

Boerjan W, Ralph J, Baucher M. 2003. Lignin biosynthesis. Annual Review of Plant Biology 54:519-546.

Bollok M, Reczey K, Zacchi G. 2000. Simultaneous saccharification and fermentation of steam-pretreated spruce to ethanol. Applied Biochemistry and Biotechnology 84(6):69-80.

Boyle M, Barron N, McHale AP. 1997. Simultaneous saccharification and fermentation of straw to ethanol using the thermotolerant yeast strain *Kluyveromyces marxianus* IMB3. Biotechnology Letters 19(1):49-51.

Brady D, Marchant R, McHale L, McHale AP. 1994. Production of ethanol by the thermotolerant yeast *Kluyveromyces marxianus* IMB3 during growth on lactose-containing media. Biotechnology Letters 16(7):737-740.

Bransby D. 2004. Switchgrass Profile. http://bioenergy.ornl.gov/papers/misc/switchgrass-profile.html.

Brownell HH, Saddler JN. 1984. Steam-explosion pretreatment for enzymatic hydrolysis. Biotechnology and Bioengineering 14:55-68.

California Energy Commission. 2004. Ethanol fuel incentives applied in the US, reviewed from California's perspective, staff report P600-04-001. Sacramento, California.

Chang VS, Burr B, Holtzapple MT. 1997. Lime pretreatment of switchgrass. Applied Biochemistry and Biotechnology 63(5):3-19.

Chang VS, Holtzapple MT. 2000. Fundamental factors affecting biomass enzymatic reactivity. Applied Biochemistry and Biotechnology 84(6):5-37.

Chang VS, Kaar WE, Burr B, Holtzapple MT. 2001. Simultaneous saccharification and fermentation of lime-treated biomass. Biotechnology Letters 23(16):1327-1333.

Chang VS, Nagwani M, Holtzapple MT. 1998. Lime pretreatment of crop residues bagasse and wheat straw. Applied Biochemistry and Biotechnology 74(3):135-159.

Chen L, Auh C, Chen F, Cheng XF, Aljoe H, Dixon RA, Wang ZY. 2002. Lignin deposition and associated changes in anatomy, enzyme activity, gene expression, and ruminal degradability in stems of tall fescue at different developmental stages. Journal of Agricultural and Food Chemistry 50(20):5558-5565.

Chung YC, Bakalinsky A, Penner MH. 2005. Enzymatic saccharification and fermentation of xylose-optimized dilute acid-treated lignocellulosics. Applied Biochemistry and Biotechnology 121:947-961.

Dale BE, Leong CK, Pham TK, Esquivel VM, Rios I, Latimer VM. 1996. Hydrolysis of lignocellulosics at low enzyme levels: Application of the AFEX process. Bioresource Technology 56(1):111-116.

Deshpande V, Sivaraman H, Rao M. 1983. Simultaneous saccharification and fermentation of cellulose to ethanol using *Penicillium funiculosum* cellulase and free or immobilized *Saccharomyces uvarum* cells. Biotechnology and Bioengineering 25(6):1679-1684.

Dien BS, Jung H-JG, Vogel KP, Casler MD, Lamb JFS, Iten L, Mitchell RB, Sarath G. 2006. Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. Biomass and Bioenergy 30(10):880-891.

Dowe N, McMillan J. 2001. SSF experimental protocols: lignocellulosic biomass hydrolysis and fermentation. Golden, CO: National Renewable Energy Laboratory.

Duff SJB, Murray WD. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. Bioresource Technology 55(1):1-33.

Edgardo A, Carolina P, Manuel R, Juanita F, Jaime B. 2008. Selection of thermotolerant yeast strains *Saccharomyces cerevisiae* for bioethanol production. Enzyme and Microbial Technology 43(2):120-123.

El-Nashaar HM, Banowetz GM, Griffith SM, Casler MD, Vogel KP. 2009. Genotypic variability in mineral composition of switchgrass. Bioresource Technology 100(5):1809-1814.

Energy Information Administration. 2007. Petroleum Basic Statistics. Washington, D.C.: http://www.eia.doe.gov/basics/quickoil.html.

Environmental Protection Agency. 2008. Renewable Fuels Standard. http://www.epa.gov/OMS/renewablefuels/ ed. Washington, D.C.: EPA.

Fan LT, Lee YH, Gharpuray MM. 1982. The nature of lignocellulosics and their pretreatments for enzymatic hydrolysis. Advances in Biochemical Engineering/Biotechnology. Manhattan, KS: Springer Berlin/Heidelberg. p 157-187.

Fleming M, Barron N, McHale L, Marchant R, McHale AP. 1993. Studies on the growth of a thermotolerant yeast strain, *Kluyveromyces marxianus* IMB3, on sucrose-containing media. Biotechnology Letters 15(12):1195-1198.

Freese E, Sheu CW, Galliers E. 1973. Function of lipophilic acids as antimicrobial food additives. Nature 241(5388):321-325.

Furlan SA, Schneider ALS, Merkle R, Carvalho-Jonas MF, Jonas R. 2001. Optimization of pH, temperature and inoculum ratio for the production of beta-D-galactosidase by *Kluyveromyces marxianus* using a lactose-free medium. Acta Biotechnologica 21(1):57-64.

Galbe M, Zacchi G. 2002. A review of the production of ethanol from softwood. Applied Microbiology and Biotechnology 59(6):618-628.

Gardner KH, Blackwell J. 1974. Structure of native cellulose. Biopolymers 13(10):1975-2001.

Ghose TK. 1987. Measurement of cellulase activities. Pure and Applied Chemistry 59(2):257-268.

Gunter LE, Tuskan GA, Wullschleger SD. 1996. Diversity among populations of switchgrass based on RAPD markers. Crop Science 36(4):1017-1022.

Heitz M, Capek-Ménard E, Koeberle PG, Gagné J, Chornet E, Overend RP, Taylor JD, Yu E. 1991. Fractionation of *Populus tremuloides* at the pilot plant scale: Optimization of steam pretreatment conditions using the STAKE II technology. Bioresource Technology 35(1):23-32.

Himmel ME, Ruth MF, Wyman CE. 1999. Cellulase for commodity products from cellulosic biomass. Current Opinion in Biotechnology 10(4):358-364.

Hitchcock AS, Chase A, United States Dept. of Agriculture, United States Bureau of Plant Industry Soils and Agricultural Engineering. 1951. Manual of the Grasses of the United States. Washington, D.C.: U.S. Dept. of Agriculture. 1051 p.

Hsu TA. 1996. Pretreatment of biomass. In: Wyman CE, editor. Handbook on Bioethanol, Production and Utilization. Washington, D.C.: Taylor & Francis.

Hsu TA, Ladisch MR, Tsao GT. 1980. Alcohol from cellulose. Chemtech 10(5):315-319.

Huang H-J, Ramaswamy S, Al-Dajani W, Tschirner U, Cairncross RA. 2009. Effect of biomass species and plant size on cellulosic ethanol: A comparative process and economic analysis. Biomass and Bioenergy 33(2):234-246.

Hughes DB, Tudroszen NJ, Moye CJ. 1984. The effect of temperature on the kinetics of ethanol production by a thermotolerant strain of *Kluyveromyces marxianus*. Biotechnology Letters 6(1):1-6.

Kashket ER. 1987. Bioenergetics of lactic acid bacteria - cytoplasmic pH and osmotolerance. Fems Microbiology Reviews 46(3):233-244.

Kim S, Dale BE. 2005. Environmental aspects of ethanol derived from no-tilled corn grain: nonrenewable energy consumption and greenhouse gas emissions. Biomass & Bioenergy 28(5):475-489.

Kiran Sree N, Sridhar M, Suresh K, Banat IM, Venkateswar Rao L. 2000. Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. Bioresource Technology 72(1):43-46.

Klemm D, Philipp B, Heinze T, Heinze U, Wagenknecht W. 1998. Comprehensive cellulose chemistry. I. fundamentals and analytical methods. Weinheim: Wiley-VCH.

Knight P. 2006. Sugar and ethanol in Brazil and South America. International Sugar Journal 108(1293):472.

Koshi PT, Stubbendieck J, Eck HV, McCully WG. 1982. Switchgrasses: forage yield, forage quality and water-use efficiency. Journal of Range Management 35(5):623-627.

Krishna SH, Chowdary GV, Reddy DS, Ayyanna C. 1999. Simultaneous saccharification and fermentation of pretreated *Antigonum leptopus* (Linn) leaves to ethanol. Journal of Chemical Technology and Biotechnology 74(11):1055-1060.

Ladisch MR, Ladisch CM, Tsao GT. 1978. Cellulose to sugars - new path gives quantitative yield. Science 201(4357):743-745.

Lark N, Xia Y, Qin C-G, Gong CS, Tsao GT. 1997. Production of ethanol from recycled paper sludge using cellulase and yeast, *Kluveromyces marxianus*. Biomass and Bioenergy 12(2):135-143.

Laser M, Schulman D, Allen SG, Lichwa J, Antal MJ, Lynd LR. 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresource Technology 81(1):33-44.

Lastick SM, Spindler DD, Grohmann K. 1983. Fermentation of soluble cello-oligosaccharides by yeasts. Wood and Agricultural Residues: Research on Use for Feed, Fuels, and Chemicals. Kansas City, MO: Academic Press. p 239-251.

Lee Y, Iyer P, Torget RW. 1999. Dilute-acid hydrolysis of lignocellulosic biomass. Advances in Biochemical Engineering/Biotechnology 65:93-115.

Lemus R, Brummer EC, Lee Burras C, Moore KJ, Barker MF, Molstad NE. 2008. Effects of nitrogen fertilization on biomass yield and quality in large fields of established switchgrass in southern Iowa, USA. Biomass and Bioenergy 32(12):1187-1194.

Lemus R, Brummer EC, Moore KJ, Molstad NE, Burras CL, Barker MF. 2002. Biomass yield and quality of 20 switchgrass populations in southern Iowa, USA. Biomass and Bioenergy 23(6):433-442.

Lin KW, Ladisch MR, Schaefer DM, Noller CH, Lechtenberg V, Tsao GT. 1981. Review on effect of pretreatment on digestibility of cellulosic materials. AIChE Symposium Series(207):102-106.

Liu C, Wyman CE. 2005. Partial flow of compressed-hot water through corn stover to enhance hemicellulose sugar recovery and enzymatic digestibility of cellulose. Bioresource Technology 96(18):1978-1985.

Lynd LR, Cushman JH, Nichols RJ, Wyman CE. 1991. Fuel ethanol from cellulosic biomass. Science 251(4999):1318-1323.

Lynd LR, Elander RT, Wyman CE. 1996. Likely features and costs of mature biomass ethanol technology. Applied Biochemistry and Biotechnology 57-58:741-761.

McCabe A, Barron N, McHale L, McHale AP. 1995. Increased efficiency of substrate utilization by exposure of the thermotolerant yeast strain *Kluyveromyces marxianus* IMB3 to electric field stimulation. Biotechnology Techniques 9(2):133-136.

McLaughlin SB, Kszos LA. 2005. Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. Biomass & Bioenergy 28(6):515-535.

McLaughlin SB, Ugarte D, Garten CT, Lynd LR, Sanderson MA, Tolbert VR, Wolf DD. 2002. High-value renewable energy from prairie grasses. Environmental Science & Technology 36(10):2122-2129.

McLaughlin SB, Walsh ME. 1998. Evaluating environmental consequences of producing herbaceous crops for bioenergy. Biomass & Bioenergy 14(4):317-324.

McMillan JD. 1994. Pretreatment of lignocellulosic biomass. In: Himmel ME, Baker JO, Overend RP, editors. Enzymatic Conversion of Biomass for Fuels Production. Washington, DC: Amer Chemical Soc. p 292-324.

Millett MA, Effland MJ, Caulfield DP. 1979. Influence of fine grinding on the hydrolysis of cellulosic materials-acid versus enzymatic. Advances in Chemistry 181:71-89.

Mitchell R, Vogel KP, Sarath G. 2008. Managing and enhancing switchgrass as a bioenergy feedstock. Biofuels, Bioproducts and Biorefining 2(6):530-539.

Mok WSL, Antal MJ. 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. Industrial & Engineering Chemistry Research 31(4):1157-1161.

Moser LE, Vogel KP. 1995. Switchgrass, big-bluestem and indiangrass. In: Barnes RF, Miller DA, Nelsen CJ, editors. Forages. 5th ed. Ames, Iowa, USA: Iowa State University Press.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology 96(6):673-686.

Narendranath NV, Thomas KC, Ingledew WM. 2001. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. Journal of Industrial Microbiology & Biotechnology 26(3):171-177.

National Research Council. 1999. Committee on Biobased Industrial Products, Biobased Industrial Products - Priorities for Research and Commercialization. National Academy Press.

Neal AL, Weinstoc J, Lampen JO. 1965. Mechanisms of fatty acid toxicity for yeast. Journal of Bacteriology 90(1):126-131.

Nieves RA, Ehrman CI, Adney WS, Elander RT, Himmel ME. 1998. Survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. World Journal of Microbiology & Biotechnology 14(2):301-304.

Nilsson U, Barron N, McHale L, McHale AP. 1995. The effects of phosphoric acid pretreatment on conversion of cellulose to ethanol at 45 degrees C using the thermotolerant yeast *Kluyveromyces marxianus* IMB3. Biotechnology Letters 17(9):985-988.

Nonklang S, Abdel-Banat BMA, Cha-Aim K, Moonjai N, Hoshida H, Limtong S, Yamada M, Akada R. 2008. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Applied and Environmental Microbiology 74(24):7514-7521.

Palmqvist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology 74(1):25-33.

Philippidis GP, Smith TK, Wyman CE. 1993. Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnology and Bioengineering 41(9):846-853.

Piper PW. 1993. Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. Fems Microbiology Reviews 11(4):339-355.

Renewable Fuels Association. 2009. Historic U.S. Fuel Ethanol Production. http://www.ethanolrfa.org/industry/statistics.

Saddler JN, Hogan C, Chan MKH, Louisseize G. 1982. Ethanol fermentation of enzymatically hydrolyzed pretreated wood fractions using *Trichoderma cellulases, Zymomonas mobilis* and *Saccharomyces cerevisiae*. Canadian Journal of Microbiology 28(12):1311-1319.

Sanderson MA, Reed RL, McLaughlin SB, Wullschleger SD, Conger BV, Parrish DJ, Wolf DD, Taliaferro C, Hopkins AA, Ocumpaugh WR and others. 1996. Switchgrass as a sustainable bioenergy crop. Bioresource Technology 56(1996):83-93.

Sarath G, Baird LM, Vogel KP, Mitchell RB. 2007. Internode structure and cell wall composition in maturing tillers of switchgrass (*Panicum virgatum*. L). Bioresource Technology 98(16):2985-2992.

Simpson J, Brady D, Rollan A, Barron N, McHale L, McHale AP. 1995. Increased ethanol production during growth of electric field stimulated *Kluyveromyces marxianus* IMB3 during growth on lactose containing media at 45 degress C. Biotechnology Letters 17(7):757-760.

Singh D, Nigam P, Banat IM, Marchant R, McHale AP. 1998. Ethanol production at elevated temperatures and alcohol concentrations: Part II - Use of *Kluyveromyces marxianus* IMB 3. World Journal of Microbiology & Biotechnology 14(6):823-834.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2004a. Determination of ash in biomass. Golden, CO: National Renewable Energy Laboratory.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2004b. Determination of structural carbohydrates and lignin in biomass. Golden, CO: National Renewable Energy Laboratory.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2004c. Determination of total solids in biomass. Golden, CO: National Renewable Energy Laboratory.

Sluiter A, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2005. Determination of extractives in biomass. Biomass Program Laboratory Analytical Procedure. Golden, CO: National Renewable Energy Laboratory.

Spindler DD, Wyman CE, Grohmann K. 1989. Evaluation of thermotolerant yeasts in controlled simultaneous saccharifications and fermentations of cellulose to ethanol. Biotechnology and Bioengineering 34(2):189-195.

Suryawati L, Wilkins MR, Bellmer DD, Huhnke RL, Maness NO, Banat IM. 2008. Simultaneous saccharification and fermentation of kanlow switchgrass pretreated by hydrothermolysis using *Kluyveromyces marxianus* IMB 4. Biotechnology and Bioengineering 101(5):894-902.

Suryawati L, Wilkins MR, Bellmer DD, Huhnke RL, Maness NO, Banat IM. 2009. Effect of hydrothermolysis process conditions on pretreated switchgrass composition

and SSF ethanol yield using *Kluyveromyces marxianus* IMB 4. Process Biochemistry 44:540-545.

Szczodrak J, Targonski Z. 1988. Selection of thermotolerant yeast strains for simultaneous saccharification and fermentation of cellulose. Biotechnology and Bioengineering 31(4):300-303.

Takagi M, Abe S, Suzuki S, Emert G, Yata N. 1977. A method for production of alcohol directly from cellulose using cellulase and yeast. In: Ghose T, editor. Proceedings of Bioconversion of Cellulosic Substances into Energy, Chemicals, and Microbial Protein. New Dehli: I.I.T. p 551-571.

Thammasouk K, Tandjo D, Penner MH. 1997. Influence of extractives on the analysis of herbaceous biomass. Journal of Agricultural and Food Chemistry 45(2):437-443.

Tukey J. 1949. Comparing individual means in the analysis of variance. Biometrics 5:99-114.

Urbanchuk J. 2006. Contribution of the ethanol industry to the economy of the United States. Prepared for the Renewable Fuels Association. Wayne, PA: http://www.ethanolrfa.org/objects/documents/1295/2006_ethanol_economic_contribution_updated_021907.pdf.

US Department of Agriculture Economic Research Service. 2005. USDA feed grains baseline, 2005-14. Washington, D.C.: USDA.

Van Walsum GP, Allen SG, Spencer MJ, Laser MS, Antal MJ, Lynd LR. 1996. Conversion of lignocellulosics pretreated with liquid hot water to ethanol. Applied Biochemistry and Biotechnology 57-58(1):157-170.

Viegas CA, Rosa MF, Sa-Correia I, Novais JM. 1989. Inhibition of yeast growth by octanoic and decanoic acids produced during ethanolic fermentation. Applied and Environmental Microbiology 55(1):21-28.

Wang M, Saricks C, Santini D. 1999. Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions. Chicago, IL: Argonne National Laboratory.

Weaver JE. 1968. Prairie plants and their environment; a fifty-year study in the Midwest. Lincoln,: University of Nebraska Press. 276 p.

Weil J, Sarikaya A, Rau SL, Goetz J, Ladisch CM, Brewer M, Hendrickson R, Ladisch MR. 1997. Pretreatment of yellow poplar sawdust by pressure cooking in water. Applied Biochemistry and Biotechnology 68(1-2):21-40.

Weil JR, Sarikaya A, Rau SL, Goetz J, Ladisch CM, Brewer M, Hendrickson R, Ladisch MR. 1998. Pretreatment of corn fiber by pressure cooking in water. Applied Biochemistry and Biotechnology 73(1):1-17.

Wiselogel AE, Agblevor FA, Johnson DK, Deutch S, Fennell JA, Sanderson MA. 1996. Compositional changes during storage of large round switchgrass bales. Bioresource Technology 56(1):103-109.

Wong KKY, Saddler JN. 1992. *Trichoderma* xylanases, their properties and application. Critical Reviews in Biotechnology 12(5-6):413-435.

Wood TM, Saddler JN, Willis AW, Scott TK. 1988. Increasing the availability of cellulose in biomass materials. Methods in Enzymology 160:3-11.

Wright JD. 1988. Ethanol from biomass by enzymatic hydrolysis. Chemical Engineering Progress 84(8):62-74.

Zhang YHP, Lynd LR. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnology and Bioengineering 88(7):797-824.

Zhou J, Wang YH, Chu J, Luo LZ, Zhuang YP, Zhang SL. 2009. Optimization of cellulase mixture for efficient hydrolysis of steam-exploded corn stover by statistically designed experiments. Bioresource Technology 100(2):819-825.

APPENDICES

SAS 9.1 Program for Tukey Test comparison of IMB 1-5 and S. cerevisiae

```
DM 'log; clear; output; clear; ';
options pageno=1;
options ls=74 ps=60;
data IMB24h;
infile "h:\Research\Thesis\Results\Statistics\Tukey w Scer\IMB24.csv" dlm=",";
input Strain$ Yield24 @@;
cards;
run;
proc print data=IMB24h;
Title 'Tukey Test of 24 h Ethanol Yield IMB 1-5 and Scer';
proc glm data=IMB24h; class Strain;
model Yield24 = Strain;
means Strain/tukey;
means Strain;
run;
data IMB48h;
infile "h:\Research\Thesis\Results\Statistics\Tukey w Scer\IMB48.csv" dlm=",";
input Strain$ Yield48 @@;
cards;
run;
proc print data=IMB48h;
Title 'Tukey Test of 48 h Ethanol Yield IMB 1-5 and Scer';
proc glm data=IMB48h; class Strain;
model Yield48 = Strain;
means Strain/tukey;
means Strain;
run;
```

```
data IMB72h;
infile "h:\Research\Thesis\Results\Statistics\Tukey w Scer\IMB72.csv" dlm=",";
input Strain$ Yield72 @@;
cards;
run;
proc print data=IMB72h;
Title 'Tukey Test of 72 h Ethanol Yield IMB 1-5 and Scer';
proc glm data=IMB72h; class Strain;
model Yield72 = Strain;
means Strain/tukey;
means Strain;
run;
data IMB96h;
infile "h:\Research\Thesis\Results\Statistics\Tukey w Scer\IMB96.csv" dlm=",";
input Strain$ Yield96 @@;
cards;
run;
proc print data=IMB96h;
Title 'Tukey Test of 96 h Ethanol Yield IMB 1-5 and Scer';
proc glm data=IMB96h; class Strain;
model Yield96 = Strain;
means Strain/tukey;
means Strain;
run;
data IMB120h;
infile "h:\Research\Thesis\Results\Statistics\Tukey w Scer\IMB120.csv" dlm=",";
input Strain$ Yield120 @@;
cards;
run;
proc print data=IMB120h;
Title 'Tukey Test of 120 h Ethanol Yield IMB 1-5 and Scer';
proc glm data=IMB120h; class Strain;
model Yield120 = Strain;
means Strain/tukey;
means Strain;
run;
data IMB144h;
infile "h:\Research\Thesis\Results\Statistics\Tukey w Scer\IMB144.csv" dlm=",";
input Strain$ Yield144 @@;
cards;
run;
proc print data=IMB144h;
Title 'Tukey Test of 144 h Ethanol Yield IMB 1-5 and Scer';
proc glm data=IMB144h; class Strain;
model Yield144 = Strain;
means Strain/tukey;
```

```
means Strain;
run;
data IMB168h;
infile "h:\Research\Thesis\Results\Statistics\Tukey w Scer\IMB168.csv" dlm=",";
input Strain$ Yield168 @@;
cards;
run;
proc print data=IMB168h;
Title 'Tukey Test of 168 h Ethanol Yield IMB 1-5 and Scer';
proc glm data=IMB168h; class Strain;
model Yield168 = Strain;
means Strain/tukey;
means Strain;
run;
```

SAS 9.1 Output for Tukey Test comparison of IMB 1-5 and S. cerevisiae

Tukey Test of 24 h Ethanol Yield IMB 1-5 and Scer 1
14:18 Friday, April 17, 2009

Obs	Strain	Yield24
1	1	44.1483
2	1	40.0248
3	1	35.6774
4	2	36.3616
5	2	39.9235
6	2	34.3619
7	3	40.6912
8	3	35.1504
9	3	28.7905
10	4	16.8604
11	4	32.0496
12	5	37.8222
13	5	27.5187
14	5	34.0673
15	Scer	32.4239
16	Scer	39.0311
17	Scer	41.3271

Tukey Test of 24 h Ethanol Yield IMB 1-5 and Scer 2
14:18 Friday, April 17, 2009

The GLM Procedure

Class Level Information

Class	Levels	Values
Strain	6	1 2 3 4 5 Scer

Number of Observations Read 17
Number of Observations Used 17

The GLM Procedure

Dependent Variable: Yield24

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	337.1038596	67.4207719	2.21	0.1265
Error	11	335.1536980	30.4685180		
Corrected Total	16	672.2575575			

R-Square	Coeff Var	Root MSE	Yield24 Mean
0.501450	15.73841	5.519830	35.07235

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Strain	5	337.1038596	67.4207719	2.21	0.1265

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	5	337.1038596	67.4207719	2.21	0.1265

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield24

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	11
Error Mean Square	30.46852
Critical Value of Studentized Range	4.82295

Comparisons significant at the 0.05 level are indicated by ***.

Strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
1 - Scer	2.356	-13.014	17.726
1 - 2	3.068	-12.302	18.438
1 - 3	5.073	-10.297	20.443
1 - 5	6.814	-8.556	22.184
1 - 4	15.495	-1.689	32.680
Scer - 1	-2.356	-17.726	13.014
Scer - 2	0.712	-14.658	16.082
Scer - 3	2.717	-12.653	18.087
Scer - 5	4.458	-10.912	19.828
Scer - 4	13.139	-4.045	30.323
2 - 1	-3.068	-18.438	12.302
2 - Scer	-0.712	-16.082	14.658
2 - 3	2.005	-13.365	17.375
2 - 5	3.746	-11.624	19.116
2 - 4	12.427	-4.757	29.612
3 - 1	-5.073	-20.443	10.297
3 - Scer	-2.717	-18.087	12.653
3 - 2	-2.005	-17.375	13.365
3 - 5	1.741	-13.629	17.111
3 - 4	10.422	-6.762	27.607
5 - 1	-6.814	-22.184	8.556
5 - Scer	-4.458	-19.828	10.912
5 - 2	-3.746	-19.116	11.624
5 - 3	-1.741	-17.111	13.629
5 - 4	8.681	-8.503	25.865
4 - 1	-15.495	-32.680	1.689
4 - Scer	-13.139	-30.323	4.045
4 - 2	-12.427	-29.612	4.757
4 - 3	-10.422	-27.607	6.762
4 - 5	-8.681	-25.865	8.503

The GLM Procedure

Level of Strain	N	Yield24 Mean	Yield24 Std Dev
1	3	39.9501778	4.2359790
2	3	36.8823479	2.8171284
3	3	34.8773616	5.9550857
4	2	24.4549663	10.7403752
5	3	33.1360616	5.2144688
Scer	3	37.5940290	4.6222893

Obs	Strain	Yield48
1	1	61.3619
2	1	61.8849
3	1	55.5318
4	2	51.1240
5	2	56.9724
6	2	57.2842
7	3	62.4903
8	3	58.3573
9	3	53.9282
10	4	55.7298
11	4	56.2896
12	5	50.3359
13	5	48.3606
14	5	62.6268
15	Scer	67.0744
16	Scer	64.8078
17	Scer	65.2780

The GLM Procedure

Class Level Information

Class	Levels	Values
Strain	6	1 2 3 4 5 Scer

Number of Observations Read	17
Number of Observations Used	17

The GLM Procedure

Dependent Variable: Yield48

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	272.1689776	54.4337955	2.88	0.0670
Error	11	208.1432802	18.9221164		
Corrected Total	16	480.3122578			

R-Square	Coeff Var	Root MSE	Yield48 Mean
0.566650	7.473866	4.349956	58.20222

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Strain	5	272.1689776	54.4337955	2.88	0.0670

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	5	272.1689776	54.4337955	2.88	0.0670

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield48

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	11
Error Mean Square	18.92212
Critical Value of Studentized Range	4.82295

Comparisons significant at the 0.05 level are indicated by ***.

Strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
Scer - 1	6.127	-5.985	18.240
Scer - 3	7.462	-4.651	19.574
Scer - 4	9.710	-3.832	23.253
Scer - 2	10.593	-1.519	22.706
Scer - 5	11.946	-0.167	24.058
1 - Scer	-6.127	-18.240	5.985
1 - 3	1.334	-10.778	13.447
1 - 4	3.583	-9.959	17.125
1 - 2	4.466	-7.647	16.579
1 - 5	5.818	-6.294	17.931
3 - Scer	-7.462	-19.574	4.651
3 - 1	-1.334	-13.447	10.778
3 - 4	2.249	-11.293	15.791
3 - 2	3.132	-8.981	15.244
3 - 5	4.484	-7.628	16.597
4 - Scer	-9.710	-23.253	3.832
4 - 1	-3.583	-17.125	9.959
4 - 3	-2.249	-15.791	11.293
4 - 2	0.883	-12.659	14.425
4 - 5	2.235	-11.307	15.778
2 - Scer	-10.593	-22.706	1.519
2 - 1	-4.466	-16.579	7.647
2 - 3	-3.132	-15.244	8.981
2 - 4	-0.883	-14.425	12.659
2 - 5	1.352	-10.760	13.465
5 - Scer	-11.946	-24.058	0.167
5 - 1	-5.818	-17.931	6.294
5 - 3	-4.484	-16.597	7.628
5 - 4	-2.235	-15.778	11.307
5 - 2	-1.352	-13.465	10.760

The GLM Procedure

Level of Strain	N	Yield48 Mean	Yield48 Std Dev
1	3	59.5928394	3.52671466
2	3	55.1268688	3.47007455
3	3	58.2585635	4.28190895
4	2	56.0096829	0.39584676
5	3	53.7744441	7.72971380
Scer	3	65.7200786	1.19621882

Obs	Strain	Yield72
1	1	60.3066
2	1	60.1740
3	1	69.3436
4	2	69.9777
5	2	66.1493
6	2	66.6318
7	3	66.2763
8	3	71.4566
9	3	65.4529
10	4	66.6100
11	4	60.8936
12	5	67.9185
13	5	60.7631
14	5	77.1996
15	Scer	80.5597
16	Scer	78.6827
17	Scer	79.1711

The GLM Procedure

Class Level Information

Class	Levels	Values
Strain	6	1 2 3 4 5 Scer

Number of Observations Read	17
Number of Observations Used	17

The GLM Procedure

Dependent Variable: Yield72

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	491.8840023	98.3768005	4.52	0.0174
Error	11	239.2032281	21.7457480		
Corrected Total	16	731.0872304			

R-Square	Coeff Var	Root MSE	Yield72 Mean
0.672812	6.789756	4.663234	68.68043

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Strain	5	491.8840023	98.3768005	4.52	0.0174

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	5	491.8840023	98.3768005	4.52	0.0174

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield72

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	11
Error Mean Square	21.74575
Critical Value of Studentized Range	4.82295

Comparisons significant at the 0.05 level are indicated by ***.

Strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Scer - 5	10.844	-2.141	23.829	
Scer - 3	11.743	-1.242	24.727	
Scer - 2	11.885	-1.100	24.870	
Scer - 4	15.719	1.202	30.237	***
Scer - 1	16.196	3.212	29.181	***
5 - Scer	-10.844	-23.829	2.141	
5 - 3	0.898	-12.086	13.883	
5 - 2	1.041	-11.944	14.026	
5 - 4	4.875	-9.642	19.393	
5 - 1	5.352	-7.633	18.337	
3 - Scer	-11.743	-24.727	1.242	
3 - 5	-0.898	-13.883	12.086	
3 - 2	0.142	-12.843	13.127	
3 - 4	3.977	-10.541	18.494	
3 - 1	4.454	-8.531	17.439	
2 - Scer	-11.885	-24.870	1.100	
2 - 5	-1.041	-14.026	11.944	
2 - 3	-0.142	-13.127	12.843	
2 - 4	3.834	-10.683	18.352	
2 - 1	4.312	-8.673	17.296	
4 - Scer	-15.719	-30.237	-1.202	***
4 - 5	-4.875	-19.393	9.642	
4 - 3	-3.977	-18.494	10.541	
4 - 2	-3.834	-18.352	10.683	
4 - 1	0.477	-14.040	14.995	
1 - Scer	-16.196	-29.181	-3.212	***
1 - 5	-5.352	-18.337	7.633	
1 - 3	-4.454	-17.439	8.531	
1 - 2	-4.312	-17.296	8.673	
1 - 4	-0.477	-14.995	14.040	

The GLM Procedure

Level of Strain	N	Yield72 Mean	Yield72 Std Dev
1	3	63.2747302	5.25622536
2	3	67.5862912	2.08506617
3	3	67.7286051	3.25469685
4	2	63.7518228	4.04207869
5	3	68.6270885	8.24109079
Scer	3	79.4711517	0.97384747

Obs	Strain	Yield96
1	1	70.1680
2	1	64.1870
3	2	73.0598
4	2	70.8008
5	2	57.5618
6	3	72.2266
7	3	80.6187
8	3	79.5992
9	4	70.2009
10	4	57.9957
11	5	66.4685
12	5	61.3204
13	5	68.8933
14	Scer	87.1078
15	Scer	85.6213
16	Scer	86.1800

The GLM Procedure

Class Level Information

Class	Levels	Values
Strain	6	1 2 3 4 5 Scer

Number of Observations Read	16
Number of Observations Used	16

The GLM Procedure

Dependent Variable: Yield96

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1070.487056	214.097411	7.01	0.0047
Error	10	305.538321	30.553832		
Corrected Total	15	1376.025377			

R-Square	Coeff Var	Root MSE	Yield96 Mean
0.777956	7.677090	5.527552	72.00062

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Strain	5	1070.487056	214.097411	7.01	0.0047

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	5	1070.487056	214.097411	7.01	0.0047

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield96

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	30.55383
Critical Value of Studentized Range	4.91202

Comparisons significant at the 0.05 level are indicated by ***.

Strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Scer - 3	8.822	-6.854	24.497	
Scer - 1	19.126	1.599	36.652	***
Scer - 2	19.162	3.486	34.838	***
Scer - 5	20.742	5.066	36.418	***
Scer - 4	22.205	4.679	39.731	***
3 - Scer	-8.822	-24.497	6.854	
3 - 1	10.304	-7.222	27.830	
3 - 2	10.341	-5.335	26.017	
3 - 5	11.921	-3.755	27.597	
3 - 4	13.383	-4.143	30.909	
1 - Scer	-19.126	-36.652	-1.599	***
1 - 3	-10.304	-27.830	7.222	
1 - 2	0.037	-17.489	17.563	
1 - 5	1.617	-15.909	19.143	
1 - 4	3.079	-16.120	22.278	
2 - Scer	-19.162	-34.838	-3.486	***
2 - 3	-10.341	-26.017	5.335	
2 - 1	-0.037	-17.563	17.489	
2 - 5	1.580	-14.096	17.256	
2 - 4	3.042	-14.484	20.569	
5 - Scer	-20.742	-36.418	-5.066	***
5 - 3	-11.921	-27.597	3.755	
5 - 1	-1.617	-19.143	15.909	
5 - 2	-1.580	-17.256	14.096	
5 - 4	1.462	-16.064	18.989	
4 - Scer	-22.205	-39.731	-4.679	***
4 - 3	-13.383	-30.909	4.143	
4 - 1	-3.079	-22.278	16.120	
4 - 2	-3.042	-20.569	14.484	
4 - 5	-1.462	-18.989	16.064	

The GLM Procedure

Level of Strain	N	Yield96 Mean	Yield96 Std Dev
1	2	67.1775246	4.22919478
2	3	67.1407944	8.37223437
3	3	77.4814941	4.57934663
4	2	64.0983070	8.63042594
5	3	65.5607352	3.86723910
Scer	3	86.3030609	0.75081841

Obs	Strain	Yield120
1	1	75.9940
2	1	63.6933
3	1	75.7634
4	2	72.4291
5	2	70.7791
6	2	69.5039
7	3	81.5811
8	3	70.8396
9	3	77.9700
10	4	65.3310
11	4	62.9357
12	5	76.0632
13	5	61.2851
14	5	78.4883
15	Scer	90.9414
16	Scer	87.6288
17	Scer	88.9342

The GLM Procedure

Class Level Information

Class	Levels	Values
Strain	6	1 2 3 4 5 Scer

Number of Observations Read	17
Number of Observations Used	17

The GLM Procedure

Dependent Variable: Yield120

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	955.401652	191.080330	6.09	0.0061
Error	11	344.917132	31.356103		
Corrected Total	16	1300.318784			

R-Square	Coeff Var	Root MSE	Yield120 Mean
0.734744	7.494646	5.599652	74.71536

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Strain	5	955.4016518	191.0803304	6.09	0.0061

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	5	955.4016518	191.0803304	6.09	0.0061

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield120

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	11
Error Mean Square	31.3561
Critical Value of Studentized Range	4.82295

Comparisons significant at the 0.05 level are indicated by ***.

Strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Scer - 3	12.371	-3.221	27.964	
Scer - 5	17.223	1.630	32.815	***
Scer - 1	17.351	1.759	32.944	***
Scer - 2	18.264	2.672	33.857	***
Scer - 4	25.035	7.602	42.468	***
3 - Scer	-12.371	-27.964	3.221	
3 - 5	4.851	-10.741	20.444	
3 - 1	4.980	-10.612	20.572	
3 - 2	5.893	-9.700	21.485	
3 - 4	12.664	-4.769	30.096	
5 - Scer	-17.223	-32.815	-1.630	***
5 - 3	-4.851	-20.444	10.741	
5 - 1	0.129	-15.464	15.721	
5 - 2	1.042	-14.551	16.634	
5 - 4	7.812	-9.621	25.245	
1 - Scer	-17.351	-32.944	-1.759	***
1 - 3	-4.980	-20.572	10.612	
1 - 5	-0.129	-15.721	15.464	
1 - 2	0.913	-14.679	16.505	
1 - 4	7.684	-9.749	25.116	
2 - Scer	-18.264	-33.857	-2.672	***
2 - 3	-5.893	-21.485	9.700	
2 - 5	-1.042	-16.634	14.551	
2 - 1	-0.913	-16.505	14.679	
2 - 4	6.771	-10.662	24.203	
4 - Scer	-25.035	-42.468	-7.602	***
4 - 3	-12.664	-30.096	4.769	
4 - 5	-7.812	-25.245	9.621	
4 - 1	-7.684	-25.116	9.749	
4 - 2	-6.771	-24.203	10.662	

The GLM Procedure

Level of Strain	N	Yield120 Mean	Yield120 Std Dev
1	3	71.8169219	7.03618200
2	3	70.9040192	1.46663701
3	3	76.7968874	5.46602138
4	2	64.1333527	1.69371859
5	3	71.9455351	9.31147015
Scer	3	89.1681342	1.66867544

Obs	Strain	Yield144
1	1	76.2102
2	1	68.1098
3	1	75.6522
4	2	71.1629
5	2	69.6230
6	2	67.4175
7	3	81.8415
8	3	80.6485
9	3	79.4900
10	4	72.2822
11	4	62.5554
12	5	65.9704
13	5	60.1957
14	5	78.0640
15	Scer	93.2007
16	Scer	91.7461
17	Scer	90.2399

The GLM Procedure

Class Level Information

Class	Levels	Values
Strain	6	1 2 3 4 5 Scer

Number of Observations Read	17
Number of Observations Used	17

The GLM Procedure

Dependent Variable: Yield144

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1291.680639	258.336128	10.57	0.0007
Error	11	268.771421	24.433766		
Corrected Total	16	1560.452060			

R-Square	Coeff Var	Root MSE	Yield144 Mean
0.827761	6.542451	4.943052	75.55353

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Strain	5	1291.680639	258.336128	10.57	0.0007

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	5	1291.680639	258.336128	10.57	0.0007

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield144

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	11
Error Mean Square	24.43377
Critical Value of Studentized Range	4.82295

Comparisons significant at the 0.05 level are indicated by ***.

Strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Scer - 3	11.069	-2.695	24.833	
Scer - 1	18.405	4.641	32.169	***
Scer - 2	22.328	8.564	36.092	***
Scer - 5	23.652	9.888	37.416	***
Scer - 4	24.310	8.921	39.699	***
3 - Scer	-11.069	-24.833	2.695	
3 - 1	7.336	-6.428	21.100	
3 - 2	11.259	-2.505	25.023	
3 - 5	12.583	-1.181	26.347	
3 - 4	13.241	-2.147	28.630	
1 - Scer	-18.405	-32.169	-4.641	***
1 - 3	-7.336	-21.100	6.428	
1 - 2	3.923	-9.841	17.687	
1 - 5	5.247	-8.517	19.011	
1 - 4	5.905	-9.483	21.294	
2 - Scer	-22.328	-36.092	-8.564	***
2 - 3	-11.259	-25.023	2.505	
2 - 1	-3.923	-17.687	9.841	
2 - 5	1.324	-12.440	15.088	
2 - 4	1.982	-13.406	17.371	
5 - Scer	-23.652	-37.416	-9.888	***
5 - 3	-12.583	-26.347	1.181	
5 - 1	-5.247	-19.011	8.517	
5 - 2	-1.324	-15.088	12.440	
5 - 4	0.658	-14.731	16.047	
4 - Scer	-24.310	-39.699	-8.921	***
4 - 3	-13.241	-28.630	2.147	
4 - 1	-5.905	-21.294	9.483	
4 - 2	-1.982	-17.371	13.406	
4 - 5	-0.658	-16.047	14.731	

The GLM Procedure

Level of Strain	N	Yield144 Mean	Yield144 Std Dev
1	3	73.3240645	4.52433605
2	3	69.4011068	1.88253417
3	3	80.6600145	1.17580268
4	2	67.4187999	6.87783438
5	3	68.0767023	9.11841797
Scer	3	91.7288928	1.48047215

Obs	Strain	Yield168
1	1	75.8358
2	1	73.8047
3	1	75.8549
4	2	71.1686
5	2	69.2457
6	2	68.9354
7	3	81.2909
8	3	80.4738
9	3	79.1947
10	4	71.8465
11	4	62.7503
12	5	75.8782
13	5	60.9122
14	5	78.4922
15	Scer	94.3743
16	Scer	94.2694
17	Scer	88.0958

The GLM Procedure

Class Level Information

Class	Levels	Values
Strain	6	1 2 3 4 5 Scer

Number of Observations Read	17
Number of Observations Used	17

The GLM Procedure

Dependent Variable: Yield168

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1164.819416	232.963883	10.05	0.0008
Error	11	255.110899	23.191900		
Corrected Total	16	1419.930314			

R-Square	Coeff Var	Root MSE	Yield168 Mean
0.820336	6.285863	4.815797	76.61314

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Strain	5	1164.819416	232.963883	10.05	0.0008

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	5	1164.819416	232.963883	10.05	0.0008

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield168

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	11
Error Mean Square	23.1919
Critical Value of Studentized Range	4.82295

Comparisons significant at the 0.05 level are indicated by ***.

Strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Scer - 3	11.927	-1.483	25.336	
Scer - 1	17.081	3.672	30.491	***
Scer - 5	20.486	7.076	33.895	***
Scer - 2	22.463	9.054	35.873	***
Scer - 4	24.948	9.956	39.941	***
3 - Scer	-11.927	-25.336	1.483	
3 - 1	5.155	-8.255	18.564	
3 - 5	8.559	-4.851	21.969	
3 - 2	10.537	-2.873	23.946	
3 - 4	13.021	-1.971	28.014	
1 - Scer	-17.081	-30.491	-3.672	***
1 - 3	-5.155	-18.564	8.255	
1 - 5	3.404	-10.005	16.814	
1 - 2	5.382	-8.028	18.792	
1 - 4	7.867	-7.126	22.859	
5 - Scer	-20.486	-33.895	-7.076	***
5 - 3	-8.559	-21.969	4.851	
5 - 1	-3.404	-16.814	10.005	
5 - 2	1.978	-11.432	15.387	
5 - 4	4.462	-10.530	19.455	
2 - Scer	-22.463	-35.873	-9.054	***
2 - 3	-10.537	-23.946	2.873	
2 - 1	-5.382	-18.792	8.028	
2 - 5	-1.978	-15.387	11.432	
2 - 4	2.485	-12.508	17.477	
4 - Scer	-24.948	-39.941	-9.956	***
4 - 3	-13.021	-28.014	1.971	
4 - 1	-7.867	-22.859	7.126	
4 - 5	-4.462	-19.455	10.530	
4 - 2	-2.485	-17.477	12.508	

The GLM Procedure

Level of Strain	N	Yield168 Mean	Yield168 Std Dev
1	3	75.1651336	1.17824627
2	3	69.7832490	1.20977844
3	3	80.3197979	1.05653958
4	2	67.2984291	6.43195743
5	3	71.7608389	9.48569149
Scer	3	92.2465116	3.59499151

SAS 9.1 Program for Tukey Test comparison of pH 4.8 and pH 5.5 SSFs

```
DM 'log; clear; output; clear; ';
options pageno=1;
options ls=74 ps=60;
data ScerpH48h;
infile "h:\Research\Thesis\Results\Statistics\Tukey 4.8 vs 5.5\ScerpH48.csv" dlm=",";
input pH$ Yield48 @@;
cards;
run;
proc print data=ScerpH48h;
Title 'Tukey Test of 48 h Ethanol Yield pH Scer';
proc glm data=ScerpH48h; class pH;
model Yield48 = pH;
means pH/tukey;
means pH;
run;
```

SAS 9.1 Output for Tukey Test comparison of pH 4.8 and pH 5.5 SSFs

Tukey Test of 48 h Ethanol Yield pH Scer 6
12:25 Wednesday, April 1, 2009

Obs	pH	Yield48
1	4.8	58.4370
2	4.8	56.3315
3	4.8	57.9289
4	5.5	67.0744
5	5.5	64.8078
6	5.5	65.2780

Tukey Test of 48 h Ethanol Yield pH Scer 7
12:25 Wednesday, April 1, 2009

The GLM Procedure

Class Level Information

Class	Levels	Values
pH	2	4.8 5.5

Number of Observations Read 6
Number of Observations Used 6

The GLM Procedure

Dependent Variable: Yield48

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	99.7384744	99.7384744	75.62	0.0010
Error	4	5.2761023	1.3190256		
Corrected Total	5	105.0145766			

R-Square	Coeff Var	Root MSE	Yield48 Mean
0.949758	1.863131	1.148488	61.64294

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pH	1	99.73847436	99.73847436	75.62	0.0010

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pH	1	99.73847436	99.73847436	75.62	0.0010

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield48

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	1.319026
Critical Value of Studentized Range	3.92649
Minimum Significant Difference	2.6036

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	pH
A	65.7201	3	5.5
B	57.5658	3	4.8

The GLM Procedure

Level of pH	N	Yield48 Mean	Yield48 Std Dev
4.8	3	57.5657966	1.09868634
5.5	3	65.7200786	1.19621882

SAS 9.1 Program for Tukey Test comparison of IMB 3 SSFs w/ varied enzyme

```
DM 'log; clear; output; clear; ';
options pageno=1;
options ls=74 ps=60;
data IMB372h;
infile "h:\Research\Thesis\Results\Statistics\Tukey IMB 3 VarEnz\IMB372.csv" dlm=",";
input EnzymeFPU$ Yield72 @@;
cards;
run;
proc print data=IMB372h;
Title 'Tukey Test of 72 h Ethanol Yield Varied Enzyme';
proc glm data=IMB372h; class EnzymeFPU;
model Yield72 = EnzymeFPU;
means EnzymeFPU/tukey;
means EnzymeFPU;
run;
```

SAS 9.1 Output for Tukey Test comparison of IMB 3 SSFs w/ varied enzyme

Tukey Test of 72 h Ethanol Yield Varied Enzyme 11
12:25 Wednesday, April 1, 2009

Obs	Enzyme FPU	Yield72
1	5	37.8155
2	5	38.6543
3	5	36.3244
4	10	56.2371
5	10	57.5149
6	10	58.6033
7	15	71.6425
8	15	72.3131

Tukey Test of 72 h Ethanol Yield Varied Enzyme 12
12:25 Wednesday, April 1, 2009

The GLM Procedure

Class Level Information

Class	Levels	Values
EnzymeFPU	3	10 15 5

Number of Observations Read	8
Number of Observations Used	8

Tukey Test of 72 h Ethanol Yield Varied Enzyme 13
12:25 Wednesday, April 1, 2009

The GLM Procedure
Dependent Variable: Yield72

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1488.169179	744.084590	639.78	<.0001
Error	5	5.815193	1.163039		
Corrected Total	7	1493.984372			

R-Square	Coeff Var	Root MSE	Yield72 Mean
0.996108	2.010589	1.078443	53.63815

Source	DF	Type I SS	Mean Square	F Value	Pr > F
EnzymeFPU	2	1488.169179	744.084590	639.78	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
EnzymeFPU	2	1488.169179	744.084590	639.78	<.0001

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield72

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	5
Error Mean Square	1.163039
Critical Value of Studentized Range	4.60166

Comparisons significant at the 0.05 level are indicated by ***.

EnzymeFPU Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
15 - 10	14.5260	11.3226	17.7294	***
15 - 5	34.3797	31.1764	37.5831	***
10 - 15	-14.5260	-17.7294	-11.3226	***
10 - 5	19.8537	16.9885	22.7189	***
5 - 15	-34.3797	-37.5831	-31.1764	***
5 - 10	-19.8537	-22.7189	-16.9885	***

The GLM Procedure

Level of EnzymeFPU	N	Yield72 Mean	Yield72 Std Dev
10	3	57.4517903	1.18435239
15	2	71.9777925	0.47416478
5	3	37.5980685	1.18003799

SAS 9.1 Program for Tukey Test comparison of Scer SSFs w/ varied enzyme

```
DM 'log; clear; output; clear; ';
options pageno=1;
options ls=74 ps=60;
data Scer48h;
infile "h:\Research\Thesis\Results\Statistics\Tukey Scer VarEnz\Scer48.csv" dlm=",";
input EnzymeFPU$ Yield48 @@;
cards;
run;
proc print data=Scer48h;
Title 'Tukey Test of 48 h Ethanol Yield Varied Enzyme Scer';
proc glm data=Scer48h; class EnzymeFPU;
model Yield48 = EnzymeFPU;
means EnzymeFPU/tukey;
means EnzymeFPU;
run;
```

SAS 9.1 Output for Tukey Test comparison of Scer SSFs w/ varied enzyme

Tukey Test of 48 h Ethanol Yield Varied Enzyme Scer 6
12:25 Wednesday, April 1, 2009

Obs	Enzyme FPU	Yield48
1	5	28.1639
2	5	29.9457
3	5	28.2831
4	10	53.6373
5	10	51.7065
6	10	51.9741
7	15	67.0744
8	15	64.8078
9	15	65.2780

Tukey Test of 48 h Ethanol Yield Varied Enzyme Scer 7
12:25 Wednesday, April 1, 2009

The GLM Procedure

Class Level Information

Class	Levels	Values

EnzymeFPU 3 10 15 5

Number of Observations Read 9
Number of Observations Used 9

Tukey Test of 48 h Ethanol Yield Varied Enzyme Scer 8
12:25 Wednesday, April 1, 2009

The GLM Procedure
Dependent Variable: Yield48

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	2098.584799	1049.292399	894.95	<.0001
Error	6	7.034756	1.172459		
Corrected Total	8	2105.619555			

R-Square	Coeff Var	Root MSE	Yield48 Mean
0.996659	2.210446	1.082802	48.98565

Source	DF	Type I SS	Mean Square	F Value	Pr > F
EnzymeFPU	2	2098.584799	1049.292399	894.95	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
EnzymeFPU	2	2098.584799	1049.292399	894.95	<.0001

The GLM Procedure

Tukey's Studentized Range (HSD) Test for Yield48

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	1.172459
Critical Value of Studentized Range	4.33902
Minimum Significant Difference	2.7126

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Enzyme FPU
A	65.7201	3	15
B	52.4393	3	10
C	28.7976	3	5

The GLM Procedure

Level of EnzymeFPU	N	Yield48 Mean	Yield48 Std Dev
10	3	52.4393307	1.04607870
15	3	65.7200786	1.19621882
5	3	28.7975532	0.99607133

CURRICULUM VITAE

Brian A. Faga

Candidate for the Degree of

Master of Science

Thesis: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF PRETREATED SWITCHGRASS USING THERMOTOLERANT IMB STRAINS OF *KLUYVEROMYCES MARXIANUS*

Major Field: Biosystems Engineering

Biographical:

Personal Data:
Born on October 21, 1981, in Chicago, IL, the son of Richard and Gayle Faga. Raised in Schererville, IN and graduated from Lake Central High School.

Education:
Received Bachelor of Science in Mechanical Engineering from Purdue University, West Lafayette, Indiana, in December, 2004.
Completed the requirements for the Master of Science in Biosystems Engineering at Oklahoma State University, Stillwater, Oklahoma in May, 2009.

Experience:
Engineering Co-op at Northrop Grumman Newport News Shipbuilding, Newport News, VA, January 2003 to August 2003, May 2004 to August 2004.
Mechanical Engineer at Sargent & Lundy LLC, Chicago, IL, January 2005 to July 2007.
Graduate Research Assistant, Oklahoma State University, Stillwater, OK, August 2007 to Present

Professional Memberships:
American Society of Agricultural and Biological Engineers, American Society of Mechanical Engineers

Name: Brian A. Faga Date of Degree: May, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF PRETREATED SWITCHGRASS USING THERMOTOLERANT IMB STRAINS OF *KLUYVEROMYCES MARXIANUS*

Pages in Study: 123 Candidate for the Degree of Master of Science

Major Field: Biosystems Engineering

Scope and Method of Study:

Simultaneous Saccharification and Fermentation of pretreated switchgrass were used to evaluate the ethanol production characteristics of five strains of thermotolerant *K. marxianus* at 45 ºC compared to *S. cerevisiae* D_5A at 37 ºC. Further experiments were performed to investigate the effects of pH and enzyme loading on SSFs with *K. marxianus* IMB 3 and *S. cerevisiae* in terms of ethanol yields and fermentation times.

Findings and Conclusions:

SSFs with the five IMB strains of *K. marxianus* converted between 67% and 80% of glucan to ethanol. However, *S. cerevisiae* D_5A at 37 ºC converted 92% of glucan to ethanol and only IMB 3 had comparable yields. Decreasing the initial pH of the SSF buffer from 5.5 to 4.8 resulted in decreased ethanol yields produced by *S. cerevisiae* D_5A from 92% to 78%. Controlling the pH of SSF with IMB 3 at 45 ºC by addition of KOH in a stirred bioreactor resulted in an increase in ethanol yield from 80% to 83% by reducing inhibition caused by acetic acid. Reducing the enzyme loading from 15 FPU/g glucan in SSFs with IMB 3 and *S. cerevisiae* D_5A resulted in significant decreases in ethanol yield.

ADVISER'S APPROVAL: Dr. Mark Wilkins